Study of fat digestion in foods as an innovative approach to adjust pancreatic enzyme replacement therapy in Cystic Fibrosis

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DOCTORAL THESIS

Presented by:

Joaquim Calvo Lerma

Supervised by:

Ana Mª Andrés Grau

Carmen Ribes-Koninckx

July 2018


UNIVERSITAT
POLITÈCNICA
DE VALÈNCIA


IIAD


Instituto de Investigación
Sanitaria La Fe


MyCyFAPP

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS PARA EL DESARROLLO

Study of fat digestion in foods as an innovative approach to adjust pancreatic enzyme replacement therapy in Cystic Fibrosis

DOCTORAL THESIS

Presented by:

Joaquim Calvo Lerma

Supervisors:

Dra. Ana Mª Andrés Grau

Dra. Carmen Ribes-Koninckx

July 2018

Dra. Ana Mª Andrés Grau, Catedrática de Universidad perteneciente al Departamento de Tecnología de Alimentos y Directora del Instituto Universitario de Ingeniería de Alimentos para el Desarrollo de la Universidad Politécnica de València,

Dra. Carmen Ribes-Koninckx, Jefe de Sección del Servicio de Gastroenterología Pediátrica del Hospital Universitari i Politècnic La Fe e Investigadora Principal del grupo de Enfermedad Celíaca e Inmunopatología Digestiva del Instituto de Investigación Sanitaria La Fe de València,

CONSIDERAN: que la memoria titulada "Study of fat digestion in foods as an innovative approach to adjust pancreatic enzyme replacement therapy in Cystic Fibrosis" que presenta D. Joaquim Calvo Lerma, para aspirar al grado de Doctor de La Universitat Politècnica de València, y que ha sido realizada bajo su dirección en el Instituto Universitario de Ingeniería de Alimentos para el Desarrollo de la Universitat Politècnica de València y en el Instituto de Investigación Sanitaria del Hospital La Fe de València, reúne las condiciones adecuadas para constituir su tesis doctoral, por lo que AUTORIZAN al interesado para su presentación.

Valencia, Mayo de 2018

Fdo.: Ana Mª Andrés Grau

Fdo.: Carmen Ribes-Koninckx

A tots amb els qui he recorregut aquest camí

A todos los que me habéis acompañado en este viaje

To all the people who have walked along with me this journey

A ma mare, la persona que més satisfeta estaria d'este resultat. Gràcies per la teua insistència sempre en que tinguerem "amor propi". Ha sigut l'alè que m'ha fet avançar.

A mon pare: per deixar-me elegir el meu camí. Per escoltar-me, per llevar-li ferro a l'asunt i pels teus consells. A ma uela Selvi: per la teua bondat infinita. Als meus germans, els amors de la meua vida, que sense saber-ho heu aportat la vostra part a esta tesi. Gràcies: Marina, per la teua ànima d'artista; Berta, pel teu esperit de superació; Joan, per la llum que desprens i m'il·lumines.

A les meues ties Ampa i Roseta, el meu refugi durant aquests anys. Algun dia, Ampa, podries escriure la novel·la d'aquesta etapa sense deixar-te ni el més mínim detall. Si la llegiren, alguns plorarien i altres riurien, o les dos coses. A tu, la meua confident i la meua guia. La meua incondicional.

A les meues ties aueles, Mila, Carmen i Amparo, les que tant han resat i tant han aportat per a que a este recorregut no li faltara de res.

Als meus randoms del town: Mar, Maria S, Maria V, Rafa, Samuel, gràcies pels bons moments. Gràcies per haver-me seguit estimant després de tantes absències. I no vos queixareu, que no vos he pegat quasi la tabarra amb el tema de la tesi!

Al meu amic Pepe, per confiar en mi. Per la teua seguretat que m'has contagiat en estos 11 anys des de la Facultat de Farmàcia i el piso de Burjassot. Per les llargues converses a distància debatint sobre la vida i el futur.

A la familia León-Ferrer, por vuestra sincera acogida siempre. Gracias Pepi, Jaime y Laura.

***

A *la creme de la creme*: los power rangers del FooDiHealth Lab. Gracias por hacerme sentir orgulloso cada vez que nos hemos encontrado trabajando juntos. Sois el equipo de trabajo ejemplar e irrepetible. "El laboratorio más bonito del mundo" como tú dices, Seba, que llegaste para enseñarnos grandes valores y humanidad. Con verdadero compañerismo como demuestras siempre, Ever. Y con la pasión por la ciencia de las que forman el trípode conmigo:

Andrea y Ary. A vosotras que habéis estado incondicionalmente a mi lado, no encuentro palabras… ILU!.

Al resto de mi familia valenciana: mis ñaños Diego y Gaby, Carol, Leidy, Clarissa. Habéis llenado de vida y de aventuras esta etapa. Os llevo conmigo. ¡Nos vemos en el matrimonio de Ary!

A mis compañeros de la planta 1 de la CPI: Mariola, Victoria, Irene, Virginia… gracias por vuestros consejos. Salva, gracias por la foto de portada!

***

A Juan Pablo Lázaro: confiaste en este proyecto desde el día que levanté el teléfono y te conté de qué iba el tema. Tu experiencia, saber-hacer y calidad humana han sido inspiradoras, ¡maestro! Y como no, necesarias para que MyCyFAPP naciera y creciera.

To Tiago and Inês: we suddenly we found ourselves in front of a huge Project to get started, with no experience at all from any of our sides. You taught me that patience and positive willingness are the most necessary “ingredients” for a fruitful and pleasant team work. And you’ve got them in high “amounts”.

To the UZ-Leuven team. Thank you for being the warmest and the perfect hosts during my last-minute stay in Leuven. Mieke, for your efficiency and clever insights. Ine, for your impeccable work and persuading ability to recruit. Trudy, for your professionality hospitality. And Kris, thank you for your open mind that got me to learn a couple of lessons. Thank you for your generosity (including even blankets and crockery). Thank you for the invaluable passion, trust and wisdom you brought to this project.

To Jacqueline Floch, Miss. Brightside of MyCyFAPP: you’ve made its stars shine.

To Jessie Hulst: you once showed me that every story worth it to tell starts with a “Research Question”. You’ve been unfailing reviewer replying to my emails after 11pm. Thank you for being my kind advisor and the very critical and strict “dose” of realism and practicality I needed so much. To all the Rotterdam team: Sandra, Sylvia and Els, thank you for your Dutch touch.

A Gema y a Toni: gracias por abrirme las puertas de ITACA-Sabien donde he podido entender un poco el mundo de las tecnologías sintiéndome como en casa.

To the MyCyFAPP consortium, the fantastic “multi- and transdisciplinary” team I’d have never dreamed to work with. Thank you for all the patience with me, and for the shared experiences that have made me grow as a “project scientific manager”.

A todos los niños y familias de las unidades de FQ que habéis participado en este proyecto. Mil gracias por la confianza depositada en este proyecto, cuyos resultados ojalá sirvan para facilitaros un poquito las cosas.

***

Al servicio de Gastro, gracias por haber cuidado de mi durante tantos años: Puri, Merche, Fátima. Isabel, mi suegri, por nuestras charlas en el laboratorio. Isabel, por despertar en mí el espíritu de “la revolta”. A Ester, Begoña, y Etna, por ser mis médicos de cabecera… y de urgencias. A Águeda, por tu pasión por el cuidado de los niños y sus familias.

Als meus companys de la setena de l’IIS La Fe. A Javi: per la teua bona voluntat i paciència; per la creació de la infraestructura informàtica que ha permès treballar amb les dades. A Viqui: sense el teu talent estadístic no tindríem els models que expliquen els resultats d’esta tesi, i ni tindríem estos gràfics tan xulos. Gràcies pel teu “interval de confiança” quan m’ensenyares algunes nocions.

A mis “hermanitas”. A Sandrita: gracias por tu frescura y por aguantar mis paranoias con tanto cariño. Ana Serra: per no haver pegat a fugir al coneixer-nos; per fer posible la comunicació dels resultats. A Natalia: por la homogenización y análisis de unas muestras no demasiado agradecidas. A Maria Roca: por el placer de trabajar a tu lado en la última etapa; por tu calidad investigadora y humana. Y a mis guerreras en la batalla del día a día, a mis “BPs”: Paula y Sara. A Paula: que sin darnos cuenta nos hemos hecho mayores después de tantos años en los que hemos aprendido a comprendernos, tu tan “práctica” y yo tan “teórico”, pasando por todas las ESPGHAN y coffee breaks (hasta podría salir una serie de todo esto). A Sara: gràcies per la teua

integritat infal·lible, pel teu bon ull per a tot i per la teua eficiència; pel tàndem de management que conformem que ha fet que tot açò avançara.

A Ana Heredia, tan cerca de esta tesis. Tan cercana, tan certera! Muchas gracias.

A Celia Martínez, mi gran descubrimiento. Te encontré en el momento idóneo. La pieza imprescindible que hizo que este proyecto sea hoy una realidad. Mi gran maestra de proyectos europeos.

***

A las que un día me crucé en su camino y consiguieron que mi vida cambiara dos veces: mis directoras. Mis maestras. Mis referentes. Ha sido un honor y un privilegio recorrer este trayecto de vuestras manos, siempre con la concesión de autonomía que me habéis dado. Mi mayor agradecimiento es hacia vosotras, para el cual no he podido encontrar mejores palabras que las que siguen.

A La Doctora (Carmen Ribes): cuando era un alumno de prácticas usted me abrió las puertas para seguir en su equipo. Y año tras año siguió apostando por mí, confiando en mi trabajo y ofreciéndome oportunidades que nunca habría soñado, las que me han convertido en el investigador que soy hoy. Gracias por los lunes por la tarde de transmisión de sabiduría y por todos los viajes que me han hecho conocer el mundo. Gracias por haberme acompañado hasta aquí.

A Ana Andrés: el pur atzar va fer que ens trobarem; mai ho podré agrair prou. La confiança que vas depositar en mi des d'eixe dia ha sigut un motor per a que m'esforçara, creguera en mi mateix i fins i tot, per a que descobrira que podia aplegar on no havia imaginat. I sempre m'has oferit el teu caliu, amb la porta el despatx oberta, on vam començar a planificar esta tesi, quan jo quasi no sabia el que era un doctorat. Durant tot este temps, gràcies per la teua vocació de professora, que m'ha fet aprendre el que hui se... i que has sabut transmetre'm. Gràcies per haver-me acompanyat fins ací.

Gràcies a tots els qui heu estat al meu costat

ABSTRACT

Pancreatic enzyme replacement therapy (PERT) is the treatment for palliating pancreatic insufficiency in Cystic Fibrosis. It consists of the exogenous administration of enzyme supplements in every meal. Up to date there is not a scientifically-valid method to individual dose adjustment. This leads to maldigestion and malabsorption, and eventually to a detriment of nutritional status and disease prognosis. The aim of this thesis was to develop a method to adjust PERT by exploring food properties under *in vitro* digestion conditions as possible determinants of lipolysis and thus of the optimal PERT dose. The secondary objective was to test the validity of the method in patients by assessing coefficient of fat absorption and identifying an individual correction factor based on individual patients' characteristics. A first stage of the research showed that very different PERT dose criteria were being applied along Europe regardless of the nutritional status of the patients. The experimental work conducted in the lab, showed that the gastrointestinal conditions during digestion are determinants of lipolysis, especially the intestinal pH and the bile salts concentration. The food properties, including interactions between nutrients, lipid structure within the food matrix and the textural properties, had a significant impact on lipolysis. The *in vitro* digestion method also revealed that the optimal PERT dose was not dependent upon fat content of food exclusively, increasing doses leading to decreased lipolysis in some foods. The modelling of these results led to the predictive equations of the theoretical optimal dose of PERT (TOD) for a selection of foods, which were tested in a pilot study setting. When patients followed the fixed test diet taking the corresponding TOD, a median coefficient of fat absorption of 90% (clinical target) was achieved and individual patients' characteristics were not significantly associated with this result, meaning that food properties were the main determinants of lipolysis. Therefore, the conclusion of this thesis is that an evidence-based method to adjust PERT in CF patients was successfully developed.

RESUMEN

La terapia de sustitución enzimática (PERT) es el tratamiento para paliar la insuficiencia pancreática en fibrosis quística. Consiste en la administración exógena de suplementos enzimáticos en cada comida. Sin embargo, hasta la fecha no hay un método científicamente válido para ajustar la dosis, lo que conlleva a maldigesitón y malabsorción de nutrientes, y eventualmente a un detrimento del estado nutricional y del pronóstico de la enfermedad. El objetivo de esta tesis fue desarrollar un método para ajustar la PERT mediante la exploración de las propiedades de los alimentos bajo condiciones de digestión *in vitro* como posibles determinantes de la lipólisis y por tanto de la dosis del suplemento enzimático. El objetivo secundario fue poner a prueba la validez del método en pacientes evaluando el coeficiente de absorción de grasa e identificando un factor de corrección individual basado en las características de los pacientes. El primer paso de la investigación mostró que se estaban aplicando criterios de dosificación muy diferentes en Europa, sin haber diferencias en el estado nutricional de los pacientes de distintos países. El trabajo experimental realizado en el laboratorio mostró que las condiciones gastrointestinales durante la digestión fueron determinantes de la lipólisis, en especial el pH intestinal y la concentración de sales biliares. Las propiedades de los alimentos, incluyendo interacciones entre nutrientes, estructura del lípido en la matriz alimento y las propiedades texturales, tuvieron un impacto significativo en la lipólisis. El método de digestión *in vitro* también reveló que la dosis óptima de PERT no era dependiente del contenido de grasa exclusivamente, ya que el aumento de la dosis en algunos alimentos condujo a la disminución de la lipólisis. La modelización de estos resultados permitió obtener las ecuaciones predictivas de la dosis óptima teórica de PERT (TOD) para una selección de alimentos, los cuales fueron testados en el contexto de un estudio piloto. Cuando los pacientes siguieron una dieta fijada con las correspondientes TOD, la mediana del coeficiente de absorción de grasa fue del 90% (objetivo clínico) y las características individuales de los pacientes no tuvieron asociación estadística con este resultado, lo que significa que las propiedades de los alimentos son el principal determinante de la lipólisis. Así pues, la conclusión de

esta tesis es que se ha desarrollado con éxito el primer método para ajustar la terapia de sustitución enzimática en fibrosis quística.

RESUM

La teràpia de substitució enzimàtica (PERT) és el tractament per a pal·liar la insuficiència pancreàtica en fibrosi quística. Consisteix en l'administració exògena de suplements enzimàtics en cada menjar. No obstant, fins a la data no hi ha cap mètode científicament vàlid per ajustar la dosi, la qual cosa condueix a la maldigestió i malabsorció de nutrients, i eventualment a un detriment de l'estat nutricional i del pronòstic de la malaltia. L'objectiu d'esta tesi fou desenvolupar un mètode per ajustar la PERT mitjançant l'exploració de les propietats dels aliments baix condicions de digestió *in vitro* com a possibles determinants de la lipòlisi i per tant de la dosi del suplement enzimàtic. L'objectiu secundari fou posar a prova la validesa del mètode en pacients, tot avaluant el coeficient d'absorció de greix i identificant un factor de correcció individual basat en les característiques dels pacients. El primer pas de la recerca va mostrar que s'estaven aplicant criteris de dosificació molt diferents a Europa, sense haver diferencies en l'estat nutricional dels pacients de distints països. El treball experimental portat a terme al laboratori va mostrar que les condicions gastrointestinals durant la digestió van ser determinants de la lipòlisi, sobre tot el pH intestinal i la concentració de sals biliars. Les propietats dels aliments, com ara interaccions entre nutrients, l'estructura del lípid en la matriu aliment i les propietats texturals, van tenir un impacte significatiu en la lipòlisi. El mètode de digestió *in vitro* també va revelar que la òptima dosi de PERT no era depenent del contingut en greix de l'aliment exclusivament, ja que l'augment de la dosi en alguns aliments va conduir a la disminució de la lipòlisi. La modelització d'estos resultats va permetre obtenir les equacions predictives de la dosi òptima teòrica de PERT (TOD) per a una selecció d'aliments, els quals van ser testats en el context d'un estudi pilot. Quan els pacients van seguir una dieta prefixada amb les corresponents TOD, la mediana del coeficient d'absorció de greix va ser del 90% (objectiu clínic) i les característiques individuals dels

pacients no van tenir associació estadística amb este resultat, la qual cosa significa que les propietats dels aliments són el principal determinat de la lipòlisi. Així doncs, la conclusió d'esta tesi és que s'ha desenvolupat amb èxit el primer mètode per a ajustar la teràpia de substitució enzimàtica en fibrosi quística.

PREFACE

Justification of the study

Cystic Fibrosis is a chronic disease affecting 35000 people in Europe. The genetic defect causes severe alterations in the respiratory and digestive system requiring patients have to stick to therapies life-long. However, whilst therapies for lung disease have been a continuous focus of research in recent years, less high-impact studies have addressed the improvement of the nutritional and pancreas-related aspects.

Pancreatic enzyme replacement therapy (PERT) consists of the exogenous administration of digestive enzymes to facilitate nutrient digestion, especially fat. However, an evidence-based dosing criterion is currently missing. The adjustment of the dose is based on the experience and clinical symptoms, and clinical trials have not been able to establish a relationship between dosage and clinical outcomes. This is a backbone in the treatment of CF, as an adequate nutritional status has clearly emerged over the last few decades with its direct relation to a better lung function and consequently a better overall prognosis and survival. However, an optimal nutritional status can only be achieved by an accurate PERT and adequate nutritional support. From the clinical practice, we identified the urgent need of investigating in this area in order to tackle the research gap that today continues making impossible to satisfactorily adjust the PERT dose.

In the light of the prolonged unsuccessful situation over the years, the hypothesis of this study is that the efficacy of PERT could be related to food properties, as recent research in the field of *in vitro* digestion studies have evidenced the influence of food chemistry and structure on nutrients' breakdown.

Thus, the motivation to conduct this doctoral thesis is to explore the mechanisms underpinning lipid digestion in cystic fibrosis, including those related to foods and to patients, in order to go a step beyond in research towards an evidence-based method to adjust PERT.

This thesis has been conducted in the framework of MyCyFAPP Project funded by the European Union Horizon 2020 Programme under Grant Agreement 643806.

Dissertation outline

A papers compendium-style thesis is being presented. The introduction compiles the definitions related to Cystic Fibrosis and Food Science along with the state of the art of these two fields in which the present thesis is framed. After exposing the current need of research, the hypothesis and the objectives are described in the form of three research questions. In order to assess the hypothesis and to accomplish the objectives, the diagrams summarising the experimental plan are provided. Then, the results are gathered into three chapters, the first one corresponding to the methodological paper describing the general project in which the conducted research was based on, and the other three chapters addressing the posed research questions. A total of 10 scientific papers conform this main section of the thesis document. Therefore, after the explanation of the results, a brief summary is provided in order to recap on the main findings. Thereafter, the conclusions of the present work are stated, followed by a short comment on possible future applications of the generated knowledge and results.

Dissemination in international Journals

As for the moment of the dissertation, 3 papers have been published in scientific journals and 7 of them are under the process of review.

Published Papers

1. Calvo-lerma, J. et al. (2017) Innovative approach for self-management and social welfare of children with cystic fibrosis in Europe : development, validation and implementation of an mHealth tool (MyCyFAPP). Br. Med. J. Open 7:e014931
2. Calvo-Lerma, J. et al. (2017). Pancreatic enzyme replacement therapy in cystic fibrosis: dose, variability and coefficient of fat absorption. Revista espanola de enfermedades digestivas, 109(10), 684-689.
3. Calvo-Lerma, J. et al. (2017). Nutritional status, nutrient intake and use of enzyme supplements in paediatric patients with Cystic Fibrosis; a European

multicentre study with reference to current guidelines. Journal of Cystic Fibrosis, 16(4), 510-518.

Papers under review or with major revision

4. Calvo-Lerma, J. et al. (2018). Children with Cystic Fibrosis present with dietary imbalances: a European multicentre comparison of food groups and origin of nutrient intake. Journal of The Academy of Nutrition and Dietetics (under review)
5. Calvo-lerma, J. et al. (2018) The role of gastrointestinal conditions on *in vitro* lipids' digestion. Clinical Nutrition (under review)
6. Calvo-lerma, J. et al. (2018) *In vitro* digestion of lipids in real foods: influence of lipid structure within the food matrix and interactions with non-lipid components. Journal of food Science (under review)
7. Calvo-lerma, J. et al. (2018) *Lipolysis of oil and butter under joint in vitro digestion of carbohydrate and protein rich food matrices.* Food and Function (under preparation)
8. Calvo-lerma, J. et al. (2018) *Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 1, in vitro study*. Journal of Cystic Fibrosis (under review)
9. Calvo-lerma, J. et al. (2018) *Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 2, in vivo validation of the in vitro model*. Journal of Cystic Fibrosis (under review)
10. Calvo-lerma, J. et al. (2018) *Association between faecal pH and coefficient of fat absorption in children with cystic fibrosis on a controlled diet and dose of pancreatic enzyme replacement therapy.* Journal of Cystic Fibrosis (Under preparation)

Dissemination in relevant international conferences

During the period in which the doctoral fellowship was developed, the generated results were presented at several high-impact national and international conferences:

1. J. Calvo-Lerma, A. Heredia and A. Andrés. *In vitro* digestion of fats in patients undergoing enzymatic replacement therapy: set-up of the method and assays on milk digestion. **4th International Conference of food digestion**. March 2015 (Naples, Italy). Poster
2. J. Calvo-Lerma, E. Masip, P. Crespo-Escobar, L. Montañana, D. Hervás and C. Ribes-Koninckx. Enzyme replacement therapy in Cystic Fibrosis: the challenge to keep it adjusted and the need of a new criterion. **48th ESPGHAN Annual Meeting**. May 2015 (Amsterdam, The Netherlands). Oral Communication
3. J. Calvo-Lerma. Innovative approach for self-management and social welfare of Cystic Fibrosis patients in Europe: development, validation and implementation of a telematics tool. **38th European Cystic Fibrosis Conference**. June 2015. (Brussels, Belgium). Lecture
4. J. Calvo-Lerma, A. Heredia, C. Ribes-Koninckx and A. Andrés. *In vitro* assessment of the influence of intestinal pH and enzyme/substrate ratio on fats digestion in Cystic Fibrosis patients. **49th ESPGHAN Annual Meeting**. May 2016. (Athens, Greece). Electronic Poster
5. J. Calvo-Lerma, J. Hulst, V. Fornés, I. Asseiceira, I. Claes, M. Garriga, et al. Nutritional status, nutrients intake and enzymatic supplements in a European Cystic Fibrosis cohort: a cross-sectional overview. **39th European Cystic Fibrosis Conference**. June 2016 (Basel, Switzerland). Oral communication.
6. J. Calvo-Lerma. MyCyFAPP: from lab to market. **Festival Pint of Science**. May 2016 (Valencia, Spain). Symposium
7. J. Calvo-Lerma. MyCyFAPP Project. **I Congress on Coordination and Management of EU-funded health research projects**. November 2016 (Valencia, Spain)

8. J. Calvo-Lerma, I. Peinado, V. Fornés, C. Ribes-Koninckx, A. Heredia and A. Andrés. A novel methodological approach to optimally adjust Pancreatic Enzyme Replacement Therapy in Cystic Fibrosis. **50th ESPGHAN Annual Meeting**. May 2017 (Prague, Czech Republic). Poster of distinction
9. J. Calvo-Lerma. MyCyFAPP Project. **40th European Cystic Fibrosis Conference**. June 2017 (Seville, Spain) – Nutrition Working Group. Oral communication.
10. J. Calvo-Lerma. *In vitro* digestion of fats to adjust Pancreatic Enzyme Replacement Therapy in Cystic Fibrosis. **40th European Cystic Fibrosis Conference**. June 2017 (Seville, Spain) – Nutrition Working Group. Oral communication.
11. J. Calvo-Lerma, I. Peinado, V. Fornés-Ferrer, C. Ribes-Koninckx, A. Heredia and A. Andrés. *In vitro* simulated digestion to study the influence of gastrointestinal conditions on lipolysis' extent. **40th European Cystic Fibrosis Conference**. June 2017 (Seville, Spain). Oral communication.
12. J. Calvo-Lerma, J. Hulst, I. Claes, I. Asseiceira, M. Ruperto, C. Colombo, et al. Comparación de la ingesta de nutrientes y alimentos entre población europea pediátrica con Fibrosis Quística: Proyecto MyCyFAPP. **XXIV Congreso de la Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica**. May 2017. (San Sebastián, Spain). Oral communication
13. J. Calvo-Lerma, I. Peinado, V. Fornés, C. Ribes-Koninckx, A. Heredia, A. Andrés. Digestión *in vitro* para ajustar la terapia de sustitución enzimática en Fibrosis Quística. **XXI Congreso Latinoamericano, XII Iberoamericano y XXX Portugués de Gastroenterología, Hepatología y Nutrición Pediátrica**. June 2017. (Oporto, Lisbon). Oral communication
14. J. Calvo-Lerma, C. Paz-Yépez, A. Asensio-Grau, I. Peinado, A. Heredia & A. Andrés. Digestibility and lipid profile of tuna and salmon under pancreatic insufficiency conditions. **31st EFFOST International Conference**. November 2017 (Barcelona, Spain). Oral communication

15. J. Calvo-Lerma. MyCyFAPP – An Innovative Approach for self-management & Social Welfare of CF Patients in Europe. **National Walsh Cystic Fibrosis Conference**. November 2017 (Cardiff, Wales). Lecture
16. J. Calvo-Lerma, J. Hulst, M. Boon, E. Masip, V. Fornés, M. Garriga, et al. A first approach for an evidence-based method to adjust PERT. **European CF Young Investigators' Meeting 2018**. February 2018 (Paris, France). Oral Communication
17. J. Calvo-Lerma. MyCyFAPP Project: new results and updates. **51st ESPGHAN Annual Meeting**. May 2018 (Geneva, Switzerland). Lecture
18. J. Calvo-Lerma. MyCyFAPP EU funded project for Cystic Fibrosis. **51st ESPGHAN Annual Meeting**. May 2018 (Geneva, Switzerland). Lecture
19. J. Calvo-Lerma, J. Hulst, M. Boon, E. Masip, V. Fornés, M. Garriga, et al. A first approach for an evidence-based method to adjust PERT in pediatric patients with Cystic Fibrosis. **51st ESPGHAN Annual Meeting**. May 2018 (Geneva, Switzerland). Poster
20. J. Calvo-Lerma, C. Paz-Yépez, V. Fornés-Ferrer, A. Asensio-Grau, I. Peinado, C. Ribes-Koninckx, A. Heredia and A. Andrés. Método de ajuste para la terapia de sustitución enzimática en fibrosis quística. Parte I estudio *in vitro*. **XXV Congreso de la Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica**. May 2017. (Granada, Spain). Oral communication
21. J. Calvo-Lerma, J. Hulst, M. Boon, E. Masip, V. Fornés, M. Garriga, et al. Método de ajuste para la terapia de sustitución enzimática en fibrosis quística. Parte II validación del estudio *in vitro*. **XXV Congreso de la Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica**. May 2017. (Granada, Spain). Oral communication
22. J. Calvo-Lerma, J. Hulst, M. Boon, E. Masip, V. Fornés, M. Garriga, et al. A first approach for an evidence-based method to adjust PERT in pediatric patients with Cystic Fibrosis. **41st European Cystic Fibrosis Conference**. June 2018

Awards

The candidate has been awarded with the following grants:

- Trainee grant to attend the COST FA 1001 - Training School in Milan "The application of Innovative Fundamental Food-structure-property Relationships to the Design of Foods for Health, Wellness and Pleasure" (2014)
- ESPGHAN Young Investigator Award to attend the 48th Annual Meeting (2015)
- ESPGHAN Young Investigator Award to attend the 49th Annual Meeting (2016)
- ESPGHAN Young Investigator Award to attend the 50th Annual Meeting (2017)
- ERASMUS PLUS Grant (2018)
- Trainee grant to attend the IT Prevent Summer School in Bertinoro (2018)
- Best short oral communication Award of the Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica at the XXV anual conference (2018)

TABLE OF CONTENTS

1. INTRODUCTION……1

1.1. Cystic Fibrosis……3

1.2. Pancreatic enzyme replacement therapy……5

1.3. Dietary intervention in Cystic Fibrosis……8

1.4. Dietary lipids……10

1.5. Food Properties……13

1.6. Food digestion……19

1.7. Factors affecting lipid digestion……22

1.8. *In vitro* digestion methods……28

References……33

2. HYPOTHESIS AND OBJECTIVES……39

3. EXPERIMENTAL PLAN……45

3.1. Multicentre study to assess PERT and nutrition in patients with Cystic Fibrosis..47

3.2. *In vitro* digestion studies……47

3.3. Pilot study to assess an evidence-based method for PERT……48

4. RESULTS……53

CHAPTER 1. HORIZON 2020 PROJECT TO FUND THE RESEARCH……44

PAPER 1. Innovative approach for self-management and social welfare of children with Cystic Fibrosis in Europe: development, validation and implementation of an mHealth tool (MyCyFAPP). ……57

CHAPTER 2. CURRENT SITUATION OF NUTRITION AND PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS CHILDREN IN EUROPE……77

PAPER 2. Pancreatic enzyme replacement therapy in cystic fibrosis: dose, variability and coefficient of fat absorption……79

PAPER 3. Nutritional status, nutrient intake and use of enzyme supplements in paediatric patients with Cystic Fibrosis; a European multicentre study with reference to current guidelines……95

PAPER 4. Children with Cystic Fibrosis present with dietary imbalances: a European multicentre comparison of food groups and origin of nutrient intake……117

CHAPTER 3. EFFECT OF FOOD INTRINSIC AND EXTRINSIC FACTORS ON LIPID DIGESTION……139

PAPER 5. The role of gastrointestinal conditions on *in vitro* lipids' digestion……..141

PAPER 6. *In vitro* digestion of lipids in real foods: influence of lipid structure within the food matrix and interactions with non-lipid components……..165

PAPER 7. Lipolysis of oil and butter under joint *in vitro* digestion of carbohydrate and protein rich food matrices. ……191

CHAPTER 4. AN EVIDENCE-BASED METHOD TO ADJUST PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS……211

PAPER 8. Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 1, *in vitro* study……213

PAPER 9. Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 2, in vivo validation of the *in vitro* model……233

PAPER 10. Association between faecal pH and coefficient of fat absorption in children with cystic fibrosis on a controlled diet and dose of pancreatic enzyme replacement therapy ……253

5. SUMMARY AND CONCLUSIONS……271

5.1. Summary of the results……273

5.2. Conclusions……277

6. FUTURE PERSPECTIVES……279

6.1. Generated basic knowledge in the field of food science……281

6.2. Clinical applications……282

6.3. MyCyFAPP Project……283

ANNEXES……285

ANNEX 1: Food record template used in the multicentre European survey on cystic fibrosis nutritional habits……287

ANNEX 2: Formulation of the four bile compositions and concentrations……291

ANNEX 3: Composition of simulated digestion fluids. The addition of pepsin, Ca2+ solution and water will result in the correct electrolyte concentration in the final digestion mixture……..292

ANNEX 4: Characteristic parameters of the log-logistic dose-response models for explaining kinetics in the three sets of experiments……..293

ANNEX 5: Classification of foods according to the lipid structure within the food matrix……..295

ANNEX 6: Protocol for the participant used in the pilot study……296

ANNEX 7: Test menu used in the pilot study……301

LIST OF FIGURES

Figure 1.1. CFTR protein acting as a chloride ion channel between inside and outside the cell. Genetic defect impairs the transport of the ion, leading to the secretion of the thick mucus that entails respiratory and gastrointestinal complications……..3

Figure 1.2. Bristol Stool Scale chart……..7

Figure 1.3. Structure of a triacylglycerol (triglyceride) including the glycerol backbone and the three fatty acid chains. In the example, three long chain fatty acids of 18 carbon atoms conform the triglyceride molecule: a polyunsaturated FA (C18:2) with the double bonds in C9 and C12 in the sn-3 position, a saturated FA (C18:0) with no double bounds in the sn-2 position and a monounsaturated FA (C18:1) with a double bound in C9 in the sn-1 position. ……11

Figure 1.4. Frequent stereospecific position of fatty acids in the triglyceride molecule in foods……13

Figure 1.5. Oil-in-water emulsion in the milk matrix……15

Figure 1.6. Enzymatic action during oral digestion. α-amylase breaks down polysaccharide chains……19

Figure 1.7. Enzymatic action during gastric digestion. Tripsin attacks protein molecules resulting in the release of peptides (up). Gastric lipase starts triglycerides digestion (down) ……20

Figure 1.8. Enzymatic action during gastric digestion. Tripsin attacks protein molecules resulting in the release of peptides (up). Gastric lipase starts triglycerides digestion (down) ……22

Figure 3.1. Diagram of the experimental plan applied to conduct the European multicentre study on nutritional habits and PERT use. This experimental plan allowed for the generation of the results reported in Chapter 2. ……49

Figure 3.2. Diagram of the experimental plan applied to conduct the *in vitro* digestion studies. This experimental plan led to the results reported in Chapter 3. ……50

Figure 3.3. Diagram of the experimental plan applied to conduct the development and validation of the evidence-based method to adjust PERT. It led to the results explained in Chapter 4. ……51
Figure 4.1. Overview of current nutritional therapies in Cystic Fibrosis and the tools needed for successfully achieving a good nutritional status, quality of life and disease prognosis……62
Figure 4.2. General overview and interrelation of work packages (WP)……65
Figure 4.3. Summary of the Project: generated tools (a), expected final scenario at the end of the Project (b) and desired outcomes (c)……69
Figure 4.4. Effect of enzyme to substrate ratio (log E/S) on Coefficient of fat absorption (CFA) according to variability on enzyme to substrate ratio intake (log SD E/S)
Figure 4.5. Boxplots of anthropometric parameters in the different CF centers: BMI SD-scores, HFA SD-scores and WFA SD-scores. BMI: body mass index for age; HFA: height for age; WFA: weight for age……103
Figure 4.6. Mean values (with 95% confidence interval) of macronutrients intake as percentage of total daily energy intake: carbohydrates (2A), protein (2B) and lipids (2C). Vertical dotted lines represent the recommended intake range or value; vertical line represents the mean value of all the centres. Total daily energy intake (2D) represented as mean daily energy intake per patient minus the minimum amount recommended (110% from the recommendation in an age-matched healthy population)……104
Figure 4.7. Dose of PERT per meal type and centre. Expressed as mean (E/S in LU/g fat) in Figure A and expressed as intra-patient variability (SD E/S) in Figure B. B, breakfast; S1, snack 1; L, lunch; S2, snack 2; D, dinner; S3, snack 3. Horizontal lines indicate the recommended range for dose in terms of lipase units per gram of fat (2000-4000 LU/g fat) according to Turck et al. 2016. ……107
Figure 4.8. Contribution of food groups to daily total protein intake in a multicentre paediatric population of children with cystic fibrosis (n= 207). Dots represent the mean

% of total protein coming from a food group/day and vertical bars represent the standard error. ……131

Figure 4.9. Contribution of food groups to daily total carbohydrates (red area) and sugar (blue area) intake in a multicentre paediatric population of children with cystic fibrosis (n= 207). Dots represent the mean % of total carbohydrates/sugars coming from a food group/day and vertical bars the standard error. ……132

Figure 4.10. Contribution of food groups to daily total fat intake (red area), and to SFA (blue area), PUFA (green area) and MUFA (yellow area) intake in a multicentre paediatric population of children with cystic fibrosis (n= 207). Dots represent the mean % of total fats coming from a food group/day and vertical bars the standard error……133

Figure 4.11. Lipolysis extent at the end of intestinal digestion (mean and standard deviation) **a)** after gastric digestion at three different pH (3, 4 and 5) and in two sets of intestinal conditions: healthy (pH7 and 10mM bile salts) and CF-affected (pH6 and 1mM bile salts); **b)** using four bile compositions (F1, bovine bile salts; F2, porcine bile salts; F3, high-glycocholic bile salts; F4, high-taurocholic bile salts) and in two concentrations of bile salts (1mM and 10mM) for F1 and F2; **c)** using three different ratios of food/simulated digestive fluids, and two concentrations of fat in the digestion medium (HFF, high fat food and LFF, low fat food) ……151

Figure 4.12. Lipolysis curves obtained with the predictive equations of the model. **a)** effect of gastric pH, and the combination of intestinal pH and bile salts concentration; **b)** effect of the bile composition and concentration; **c)** effect of the ratio of digestive fluid/food and the fat concentration of the food sample. ……154

Figure 4.13. Heatmap representing lipolysis extent of the 52 assessed foods under different combinations of intestinal conditions pH/bile concentration at the PERT dose of 2000 LU/g fat……173

Figure 4.14. Nutrient interaction and the effect on lipolysis: protein-lipid interaction (a), protein-SFA interaction (b) and starch-lipid interaction (c). *SFA, saturated fatty acids*……177

Figure 4.15. Boxplots representing lipolysis extent of the assessed food groups according to the lipid structure in the food matrix, under the digestion conditions of intestinal pH6 and bile salts concentration 1mM……..183
Figure 4.16. Boxplots representing lipolysis extent of the assessed food groups according to the lipid structure in the food matrix, under the digestion conditions of intestinal pH7 and bile salts concentration 10mM……184
Figure 4.17. Viscosity conferred to the digestion media by the food matrices individually and when combined with each other. Viscosity is expressed in distance covered by the digestion fluid. ……..202
Figure 4.18. Free fatty acids profile of butter and olive oil when digested with bread, fresh cheese, hake, potato and turkey. ……..207
Figure 4.19. Free fatty acids profile of olive oil when digested with a carbohydrate matrix (bread or potato) and a protein matrix (fresh cheese, hake or turkey)……208
Figure 4.20. Free fatty acids profile of olive oil when digested with a carbohydrate matrix (bread or potato) and two protein matrices (fresh cheese and hake or turkey)……..209
Figure 4.21. Lipolysis extent (%) versus different PERT dose (LU/g lipid) after *in vitro* digestion (intestinal pH 6 and 1 mM bile concentration). The horizontal dotted line represents a 90 % of lipolysis extent target. Figure 4.21 A: foods with an increasing lipolysis extent with dose; Figure 4.21 B: foods with a decreasing lipolysis extent with dose……..221
Figure 4.22. Lipolysis extent (%) as a function of PERT dose (LU/g lipid) predicted for each food by applying the segmented mixed regression model. The dotted line represents the evolution considering all the foods together and the solid line shows the estimation for each food individually. Figure 4.22 A: foods with an increasing lipolysis extent with dose; Figure 4.22 B: foods with a decreasing lipolysis extent with dose……..222

Figure 4.23. Overview of the combined *in vitro* and *in vivo* approach to validate a new evidence-based predictive model to optimally adjust PERT dose, adapted to foods' and patients' characteristics……237

Figure 4.24. Overview of the study. The study started at day 1 afternoon with a washout diet including a fixed PERT dose (snack 2) and the administration of the red marker. From then on, and during day 2 patients followed the test diet with the fixed PERT dose, until the afternoon snack on day 2, that was taken in combination with the blue markers. The dinner of day 2 and the breakfast of day 3 conformed the washout diet after the test menu, also with a fixed PERT dose. Faeces had to be collected from the moment of the red colour intake until the first blue-dyed deposition was found. For the faeces analysis, researchers selected the samples comprised from the 1st red deposition to the 1st blue. ……238

Figure 4.25. Results of the coefficient of fat absorption (CFA) obtained in the participating centres. The horizontal dotted bar represents the median CFA of the total population, coinciding with the clinical target of 90% . Boxplots represent the median and the 1st and 3rd quartiles in all the centres. ……245

Figure 4.26. Representation of number of faeces per patient and centre and the Bristol stool scale type assigned to each individual deposition by the patient or caregiver (one patient per row). Faeces samples correspond to the study period, from the red to the blue coloured deposition. ……245

Figure 4.27. Heatmap displaying the free fatty acids profile in faeces in the participating centres. Columns represent the patients and rows the free fatty acids. The colour scale indicates the presence (amount). ……262

LIST OF TABLES

Table 1.1. Classification of the main fatty acids present in foods according to the carbon chain length and the degree of saturation/unsaturation……12

Table 1.2. Examples of natural food matrices……14

Table 1.3. Examples of supramolecular structure in some food matrices……16

Table 1.4. Lipids classification according to their structure within the food matrix according to Michalski et al. (2017) ……17

Table 1.5. Summary of factors affecting digestion in Cystic Fibrosis……28

Table 1.6. Summary of the digestion conditions to conduct an *in vitro* digestion study according to the harmonised standard protocol proposed by Minekus et al. (2014)……30

Table 1.7. Analytical determinations attached to the process of *in vitro* digestion to monitor and assess lipolysis)…… ……31

Table 4.1. List of Participating Organisations in MyCyFAPP Project……60

Table 4.2. Clinical and analytical data of patients along the study period……85

Table 4.3. Results obtained by means of the 4-day food record……86

Table 4.4. Results of the lineal regression model: effect of the variables assessed on the coefficient of fat absorption (CFA)……87

Table 4.5. Clinical and demographic characteristics of the study cohort……102

Table 4.6. Doses of PERT in the study cohort……105

Table 4.7. Food groups and subgroups classification according to the common consensus criteria. All the foods listed in the nutritional composition databases were assigned with a food group and subgroup. Relative contribution foods to macronutrients intake was made on the basis of this classification……122

Table 4.8. Daily intake* of macro- and micronutrients of children with Cystic Fibrosis in 6 European centers……105

Table 4.9. Median daily frequency of consumption of food groups among children with Cystic Fibrosis in 6 European centres as assessed by means of a 4-day weighted food record. The 13 food groups were established by applying a common consensus criterion, which were assigned to the food items included in the national nutritional composition databases used.……128

Table 4.10. Experimental design: combination of the gastrointestinal conditions as study variables and resulting fat and enzyme concentrations in the digestion medium…..……146

Table 4.11. Linear mixed regression models to assess the effect of the gastrointestinal conditions on lipolysis. ……151

Table 4.12. Linear mixed regression models showing the influence of nutritional composition on lipolysis: interaction between protein and lipids, interaction of type of fatty acid with the protein content and the interaction between starch content and lipids.……175

Table 4.13. Linear mixed regression model explaining the effect of the lipid structure in the food matrix on lipolysis at the digestion conditions of intestinal pH6 and bile concentration 1mM……180

Table 4.14. Linear mixed regression model explaining the effect of the lipid structure in the food matrix on lipolysis at the digestion conditions of intestinal pH7 and bile concentration 10mM……181

Table 4.15. Characterisation of the foods assessed: type of food matrix and nutrient composition……194

Table 4.16. Experimental design. Combination of the naturally fat-free food matrices with the type of added fat. ……196

Table 4.17. Textural properties of the naturally fat-free food matrices as measured by a TPA double compression assay. Values expressed as mean (SD)……200

Table 4.18. Influence of carbohydrates and protein content, hardness and viscosity of the gastric digestion medium on total FFA released after intestinal digestion……201

Table 4.19. Linear mixed regression model for food extrinsic factors: influence of intestinal pH and bile concentration on lipolysis extent……220
Table 4.20. Summary of lipolysis extent at the change point and theoretical optimal dose (TOD) to obtain lipolysis extent of 90%.....224
Table 4.21. Patients' characteristics and results of the pilot study……244
Table 4.22. Beta regression model to assess the influence of the study variables on CFA, including the dose of enzymes (TOD) and the individual factors intake of proton pump inhibitors (PPI), age and transit time……246
Table 4.23. Demographic and clinical data of the patients and results of the analysis of the faeces samples……259
Table 4.24. Linear regression models to explore the association between pH of faeces and the fat excretion parameters: total fat, total free fatty acids and CFA……260
Table 4.25. Linear mixed regression model explaining the association between age, transit time and use of proton pump inhibitors on the faces samples pH value……260
Table 4.26. Total amount of individual free fatty acids in faeces referred to faeces sample weight and to gram of undigested fat……261
Table 4.27. Multivariate regression model explaining the effect of age, transit time and use of PPIs on the release of C16:0, C18:0, C20:0 and C18:1……263

1. INTRODUCTION

1. INTRODUCTION

1.1. CYSTIC FIBROSIS

Cystic Fibrosis (CF) is the most common life-threating autosomal inherited disease in Europe incidence varying between 1:1300 (Ireland) and 1:25000 (Finland) new-borns. Currently over 35000 cases of CF are registered in Europe [1].

The disease is characterised by a mutation in the gene that codifies the *CFTR* protein, which acts as an ion channel located at the apical cell membrane. Currently, there are more than 2000 mutations described causing CF, being the F508del in homozygosis the most prevalent and the most severe. Defective CFTR function impairs chloride ion transport, that leads to the excessive secretion of a thick mucus by different organs [2] (**Figure 1.1**). These secretions initiate the pathophysiologic cascade leading to clinical manifestations of the disease. As CFTR is expressed in many organs, CF is a multisystem disease, especially affecting the lungs and the pancreas. The clinical manifestations include, among chronic lung disease, liver disease or salt loss syndromes, pancreatic insufficiency in 85% of the patients [3].

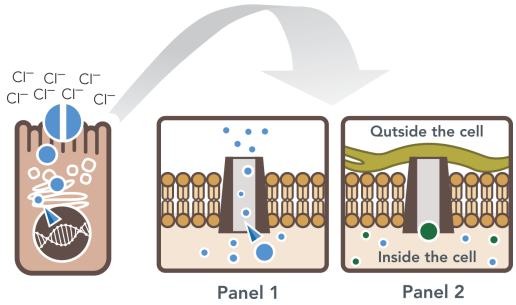


Figure 1.1. CFTR protein acting as a chloride ion channel between inside and outside the cell. Genetic defect impairs the transport of the ion, leading to the secretion of the thick mucus that entails respiratory and gastrointestinal complications

Pancreatic insufficiency

The thick mucus blocks the pancreatic duct, that impedes the secretion of the pancreatic juice, containing the pancreatic digestive enzymes, to the duodenum [4]. As a

consequence, the breakdown of nutrients, especially in lipids, is impaired [5]. While carbohydrates and protein reach normal digestion extents, lipids are the nutrient whose digestion is more dependent on pancreatic enzymes action. The lack of lipolysis, makes lipid absorption impossible, and produces the loss of lipids and liposoluble vitamins with the faeces. Not absorbing lipids is translated in the loss of important source of energy [6]. On the other hand, the obstruction of the pancreatic duct also prevents the secretion of bicarbonate, which in a normal digestion is the main agent responsible for the pH increase at this phase. The delay in the neutralisation of the acidic chyme coming from the stomach, and the lower overall pH achieved in the intestine, is another factor hindering the action of pancreatic enzymes, whose activity is optimal when close to neutrality [7,8]. Eventually, this situation leads to a weight and growth stunting in children with CF, if not correctly addressed [9].

Treatment of CF

The combination of early diagnosis and early intervention have proved to slow progression of disease and alter the clinical course of CF in future [10]. Therefore, the need of achieving an optimal treatment of the disease is crucial to start at very early stages of life, i.e. the paediatric age. Antibiotics, physiotherapy and nutrition are considered the three pillars in the treatment of CF according to the most recent standards of care [11]. In the recent years, therapies for lung disease and genetic modulators have been a continuous focus of research, but little high-impact studies have addressed the improvement of the nutritional and pancreas-related aspects [12]. The present thesis focuses on these last, which are those unequivocally associated to a better lung function and thus, to a better disease prognosis and survival.

Nutrition therapy relies on two strategies: dietary intervention and follow-up and the pancreatic enzyme replacement therapy, both in order to achieve and maintain an adequate nutritional status. This goal is, however, a challenge in most patients with CF. This has several reasons, e.g. the increased energy requirements secondary to chronic inflammation and pancreatic insufficiency on one side and the decreased

energy intake and loss of nutrients due to maldigestion and malabsorption on the other side. Therefore, the main nutritional goal in children with CF is to avoid inadequate nutrient intake and to follow a normal growth pattern according to age. Nutritional follow-up and intervention is essential and should aim at achieving an optimal adjustment of pancreatic enzyme replacement therapy (PERT) to correct pancreatic insufficiency as well as at prescribing a balanced diet according to nutritional needs [13].

The recently published guidelines on nutrition care for infants, children and adults with CF compile the most recent scientific evidence and establish recommendations regarding nutritional goals and PERT dosage [13]. The pancreatic enzyme replacement therapy and the nutritional aspects are explained in detail in the coming sections **1.2** and **1.3**.

1.2. PANCREATIC ENZYME REPLACEMENT THERAPY

In order to palliate pancreatic insufficiency, pancreatic enzyme replacement therapy (PERT) is the treatment patients have to adhere to [14,15]. PERT consists of the exogenous intake of capsules containing porcine-origin pancreatin. Pancreatin is a mix of digestive enzymes including amylase, proteases and especially lipase. The enzymes are presented in the format of granules which are covered by a gelatine film resistant to gastric pH, and dissolved at the more alkaline duodenal pH, so that at that stage of the digestion process, they can exert their hydrolytic function on nutrients [16].

Adjustment of PERT dose

The enzyme supplements are available in different sizes or doses. The unit for quantifying the dose is referred to the enzymatic activity of lipase, namely lipase units (LU). There are four sizes available in Europe: 5000, 10000, 25000 and 40000 LU.

Patients with CF and exocrine pancreatic insufficiency have to take the enzyme supplements in every meal in order to enable for food digestion, correcting this way the defective pancreatic secretion. The dietician or gastroenterologist following-up the patients at the CF unit is in charge of establishing the recommended dose, according to

the symptoms and the evolution. Normally, consensus is to take a certain amount in the main meals and half of this amount in the snacks, as long as the daily intake does not exceed the recommended maximum of 10000 LU/ g of fat. The nutrition guidelines in CF also recommend that the enzymes are supplemented in the range of 2000 to 4000 LU/g fat and meal, although acknowledging a very low degree of scientific evidence for the recommendation [12]. In addition, estimating the amount of fat a food or meal contains can get to be a difficult task for the patient, what eventually leads the regular practice to stick to the "x dose in main meals, x/2 in snacks".

Assessment of PERT dose

In order to assess the adequacy of the dose of PERT, a test of fat in stools is recommended as the golden standard, which should be performed at least once a year. It consists on the collection of faeces during 72h at the same time a food record is completed during that period of time. Then, total fat in diet is calculated, and fat amount in faeces is analysed by infrared spectrometry [17]. With this information, the coefficient of fat absorption (CFA) is calculated according to **Equation 1**.

Equation 1. $$CFA\ (\%) = \frac{fat\ in\ diet\ (g) - fat\ in\ faeces\ (g)}{fat\ in\ diet\ (g)} \cdot 100$$

The clinical target for CFA is 90%. According to this value, patients who obtain a lower result are recommended to increase the dose of PERT, whilst if the target is reached the regular dose is advised to be maintained.

Another strategy to indirectly estimate the pertinence of the dose of enzymes is based on faeces consistency. An excess of enzymes causes constipation and not administrating enough dose leads to diarrhoea [18]. The Bristol Stool Scale is a tool that can help to identify episodes of altered depositions to inadequate doses of PERT (**Figure 1.2**).

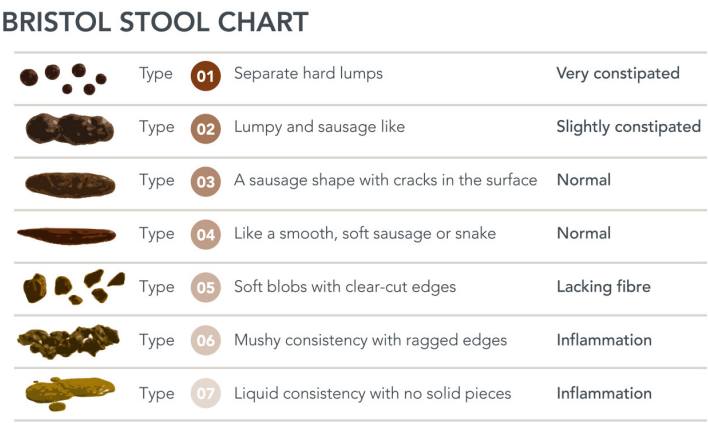


Figure 1.2. Bristol Stool Scale chart

Limitations of PERT

Despite the CFA assessment, which normally is performed only once a year, and the more daily-basis assessment of stools consistency, the optimal adjustment of the PERT of dose remains a utopia: the reason is that patients keep on facing adverse events episodes related to the dose of PERT. In fact, to our knowledge there is no scientific research that has been able to successfully explain the role of PERT dose and nutrition and their impact on the clinical outcomes. Several studies have addressed the assessment of the influence of PERT dose on CFA, but none of them have reached consistent conclusions: PERT dose is not associated with body mass index, age, type of mutation, lung function or CFA [19-21]. Focusing on the study designs, however, these studies have been conducted at an *ad libitum* diet. Therefore, they have not considered the potential impact of the type of food on the digestion process, and thus on the efficacy of PERT.

There are studies indicating the food characteristics as important factors affecting the role of pancreatic enzymes activity [22-25], while some others place the focus on the gastrointestinal conditions as the main determinants [8, 26-28]. As will be explained later, most of these studies have been conducted on the framework of isolated conditions, for example, monocomponent systems or ideal emulsions, and none of them have addressed the integral complexity of the digestion scenario composed by

the gastrointestinal environment characteristics, the specific enzymes sources enzymes (i.e. PERT) and the interactions occurring with the food matrices.

Thereby, today there is no scientific knowledge on the behaviour of PERT under digestion of foods in CF, and the recommendations of the expert guidelines rely on very low level of evidence.

1.3. DIETARY INTERVENTION IN CYSTIC FIBROSIS

As previously mentioned, the main goal of the nutrition therapy in CF is to achieve and maintain an optimal nutritional status, especially in children and adolescents [13]. This concept is defined as having a weight, height and body mass index (BMI) z-score of 0, which means equivalent to the age and gender matched healthy general population. A z-score represents the distance of a given value to the mean value measured in a given population. A z-score equal to 0 means an average value, while a z-score of -1 means the value is one standard deviation below the mean value of the reference population.

Energy and Nutrient recommendations

The dietary intervention is essential to contribute to the nutritional status goal. It consists on the recommendation of a high-calorie and high-fat diet in order to compensate the augmented energy needs caused by the pathophysiologic cascade. The infections, lack of appetite, loss of nutrients due to maldigestion and malabsorption finally cause increased respiratory rates, which imply high energy demands [14,15].

The current guidelines for nutrition in CF recommend that the energy intake should be 110-200% of the recommended energy intake of healthy age-matched population [13]. The reasons for having such a large range is that there are different complications causing high energy needs: from mild pancreatic insufficiency to intravenous antibiotics treatment. For macronutrient intake the recommendations are 20% of total daily energy intake from protein, 35-40% from lipids, and 40-45% from carbohydrates [13].

Strategies to achieve the nutritional goals

In order to achieve the high energy and fat intake goals, patients have been traditionally advised to fortify their regular meals with energy and fat-dense ingredients, such as cream, oil, cheese, etc. Another strategy includes the substitution of some low-energy foods like fruit by others, such as cakes or dairy desserts. Frequently, food choices derive in the use of processed products, which present an unhealthy nutritional profile, by including high contents of saturated fat and sugar.

However, the life expectancy of patients with CF is increasing steadily, mainly due to medical advances [29,30]. CF has now become a chronic disease that continues into adulthood [31]. Thus, the appearance of complications related to unhealthy nutrition, such as obesity or increased cardiovascular disease risk, and other age-related conditions associated with the high consumption of saturated fat and sugar have been growing [30, 32, 33]. This shift in the disease prognosis made it necessary to become cautious about the nutrient recommendations. In this sense, the guidelines currently advise the ad-libitum consumption of high fat foods when weight gain is necessary. Reaching a balanced and a lifelong healthy food behaviour and preference to fats with unsaturated fatty acids is highlighted as an upmost importance goal [13].

In fact, a recent systematic review that focused on the historical perspective of dietary intake studies in children with CF (1969-2016), showed that the nutrition dogma has to be extended from "nutrition for growth and survival" to "nutrition for health and wellbeing" [34]. The authors also identified a very limited information about nutrient intake and food choices and dietary patterns in patients with CF, claiming for contemporary information in dietary intake in the CF population.

To sum up, the nutritional intervention in CF is currently based on four objectives:

- ✓ Adequate PERT dose in every meal: 2000-4000 LU/g fat
- ✓ Nutrient intake distribution: 20% protein, 40% carbohydrates, 40% fat
- ✓ Nutritional status: BMI, height and weight z-score = 0
- ✓ Healthy food choices: avoid high intakes of sugar and saturated fat

Limitations of the dietary intervention

The dietary support and follow-up is an essential strategy to reach the nutritional status goals. However, it only occurs every three months in most of the cases. Therefore, if an incorrect nutritional behaviour or an inadequate enzyme dosage occurs, it will most likely not be detected and corrected until the next contact or visit to the CF centre. Consequently, one of the ultimate goals of the medical care in CF – achieving and maintaining an adequate nutritional status – will be jeopardised rather than potentiated. Nutritional education and treatment in CF can be, thus, considered as an ideal target to be self-managed by the patients by means of mHealth technologies.

1.4. DIETARY LIPIDS

Lipids are the most important nutrient in the diet of a patient with CF, since they provide 9 kcal/g as compared to the 4 kcal/g of carbohydrates and protein, and achieving high energy intake is one of the priorities of the treatment of the disease in order to guarantee a good nutritional status [13]. Due to exocrine pancreatic insufficiency, lipids are the nutrient whose digestion is more compromised in CF, and thus lipids are the target of investigation of the present thesis. Thus, extended explanations on lipid structure and lipid digestion are provided.

Approximately 97% of dietary lipids are triglycerides, a small percentage are represented by phospholipids, nearly 1% is cholesterol and less than 1% is constituted by different lipophilic species such as phytosterols, liposoluble vitamins and carotenoids [35]. While the common characteristic of all the lipid species is that they are insoluble in aqueous media, the structure of lipids is highly variable, and so are their physicochemical properties [36]. Thus, for example, triglycerides can be found in the form of a) droplets or vacuoles, b) stable emulsions (milk, cream, meat derivatives), c) liquid (vegetal oils, fish oils), d) solid and in crystal form (butter, palm oil, saturated fats) or e)

lipoproteins (egg yolk). Before going more in depth in lipid presence in foods, the molecular explanation of this nutrient is provided in the coming paragraphs.

Molecular structure of lipids

Triacylglycerols (TAGs) consist of three fatty acids esterified to a glycerol backbone (**Figure 1.3**). The nature of the constituent fatty acids (chain length and degree of unsaturation) and their positional distribution will dictate their physical properties and digestibility [37]. Their interaction with other components of the food matrix will also play a crucial role in the process of digestion. The intramolecular structure of TAGs corresponds to the position, or regiodistribution, of the fatty acid chains on the glycerol backbone, according to the stereospecific number (sn). The sn-1 and sn-3 are the external positions and the sn-2 is the internal. The chain length and degree of saturation/unsaturation of the fatty acids is related to the origin of the food. A saturated fatty acid is defined as a chain with no double bonds, while unsaturated fatty acids contain a double bond (monounsaturated) or more (polyunsaturated) [37].

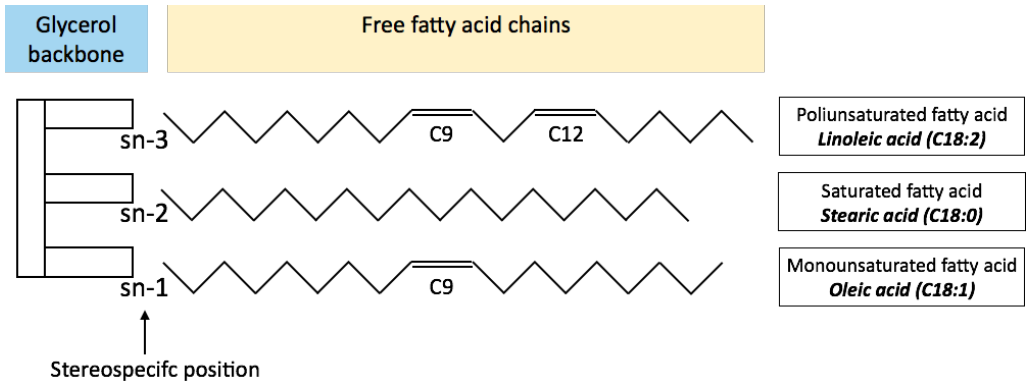


Figure 1.3. Structure of a triacylglycerol (triglyceride) including the glycerol backbone and the three fatty acid chains. In the example, three long chain fatty acids of 18 carbon atoms conform the triglyceride molecule: a polyunsaturated FA (C18:2) with the double bonds in C9 and C12 in the sn-3 position, a saturated FA (C18:0) with no double bounds in the sn-2 position and a monounsaturated FA (C18:1) with a double bound in C9 in the sn-1 position.

As shown in **Figure 1.4**, different natural fats or oils contain basically the same major fatty acids that are differently distributed within the glycerol backbone [38]. The fatty acid distribution within naturally-occurring TAGs is not random [39,40]. For example, palmitic acid is preferentially located on the sn-2 position in milk fat and lard while it is concentrated on the sn-1,3 positions in beef tallow, soybean oil and cocoa butter. Unsaturated FA (oleic, linoleic) are mainly located on sn-2 position in soybean oil and cocoa butter while in lard, oleic acid is mostly on external positions [41]. Overall, lipids in food are composed of medium and especially long chain fatty acids, as summarised in **Table 1.1**.

Table 1.1. Classification of the main fatty acids present in foods according to the carbon chain length and the degree of saturation/unsaturation

	Saturated	**Unsaturated**
Medium chain fatty acids (<10 carbon atoms)	C8:0 (caprlylic acid) C10:0 (capric acid)	-
Long-chain fatty acids (>10 carbon atoms)	C12:0 (lauric acid) C14:0 (myristic acid) C16:0 (palmitic acid) C18:0 (stearic acid) C20:0 (arachidic acid) C22:0 (behenic acid)	C18:1 (oleic acid) C18:2 (linoleic acid)

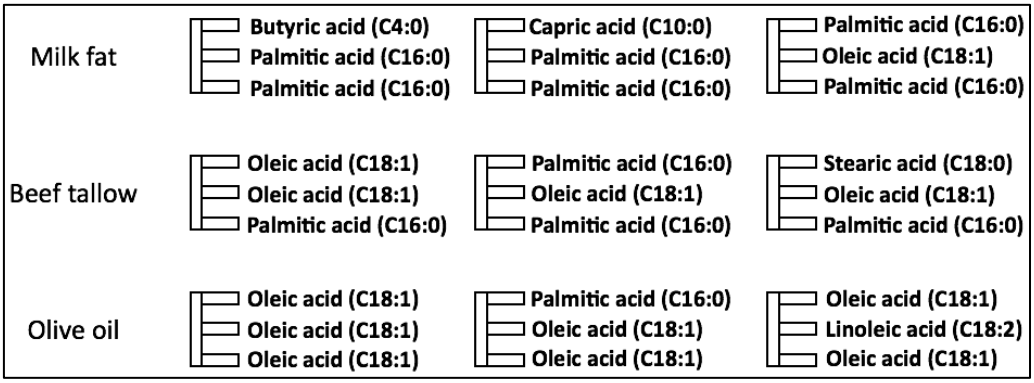


Figure 1.4. Frequent stereospecific position of fatty acids in the triglyceride molecule in foods

1.5. FOOD PROPERTIES

From a dietetic point of view foods are the sum of the nutrients they contain for their implication on nutrition and metabolism. However, from a food science perspective the concept of foods is made from a wider scope. Foods are very complex materials integrated by nutrient and non-nutrient components such as carbohydrates, protein, fat, vitamins, minerals and fibre [37]. **The matrix of a food** is defined as the organisation of its constituent molecules at multiple spatial length scales. It can be defined as the spatial architecture resulting from the assembly of macromolecules such as proteins, polysaccharides and lipids into a coordinated network [42]. Most foods are complex, heterogeneous materials composed of structural elements or domains existing as solids, liquids and/or gases. The structure of all foods is provided by nature or imparted during processing and preparation [42]. From this structure, some properties are derived, such as thermochemical or physicochemical, including texture and viscosity. The food matrix also plays a vital role in how food interacts with the gastrointestinal tract and the resulting release and uptake of nutrients [43,44].

Types of food matrix

There is a series of components that form food matrices: cell walls, starch granules, polysaccharide chains, proteins, fat droplets, fat crystals, gas bubbles, fibres. Depending on the food origin, these components can form different structures [45] as shown in **Table 1.2**.

Table 1.2. Examples of natural food matrices

Food matrix	Description	Examples
Fibrous structures	They are protein hierarchically organised to form tissues with a specific functionality that are maintained together thanks to specific interfacial interactions	Meat, fish
Vegetal-origin fleshy materials	Composed by hydrated cells that are united by the cell wall	Fruit, vegetables
Vegetal-origin encapsulated embryos	They contain a dispersion of starch, protein and lipids which are organised in packages	Grains, legumes
Emulsions	Fluid complex that contains protein, carbohydrates and lipids in a dispersion form	Milk, dairy products

In processed foods, the vast majority of fats and oils are present as emulsions. Contrary to bulk fats, emulsions consist of two immiscible liquids with one phase dispersed within the other as droplets (**Figure 1.5**). In foods, most emulsions consist of oil droplets dispersed in a continuous aqueous phase, where vegetable oil is dispersed as droplets in solution. Emulsifiers such as proteins and small-molecule surfactants such as lecithin

are used to emulsify and coat the dispersed oil droplets, which slows their separation [46].

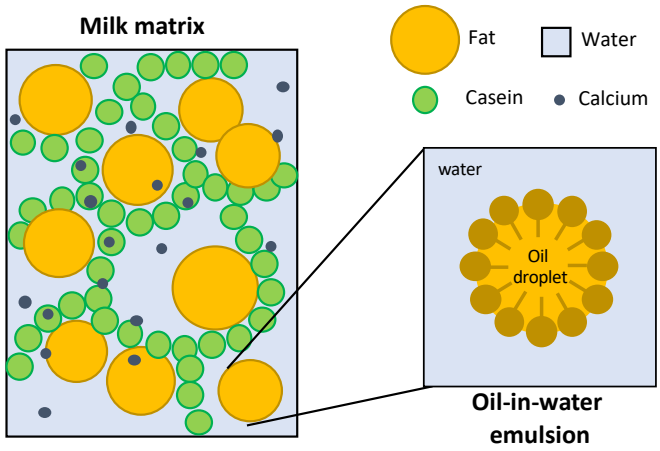


Figure 1.5. Oil-in-water emulsion in the milk matrix

Classification of lipid structures in food matrices

After providing an explanation about lipid molecular structure and having defined the complexity of the food matrix, this section focuses on the lipid classification within the food matrix. Michalski et al. in 2013 published a review addressing the structure of lipid in foods [38]. Authors defined that lipid can be classified according to two levels of complexity: supramolecular structure and macroscopic scale.

Supramolecular Structure

The supramolecular structure refers to the chemical system in which lipids are interacting with other food components. **Table 1.3** provides explanations for the systems formed in some natural foods.

Table 1.3. Examples of supramolecular structure in some food matrices according to Michalski et al. (2013) [38]

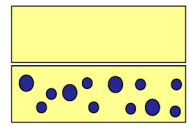
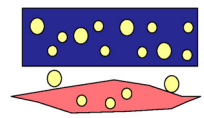
Organisation	Description
Meat and meat products	In meat, TAG are mainly present in the adipocytes, that form the adipose tissue. TAG can also be found in the muscles in the form of isolated adipocytes or droplets and within cell muscles. In processed meat, these fats are partly crystallised at ambient temperature, and sometimes even at body temperature, due to the presence of more than 40% of long-chain saturated FA, palmitic acid being mainly located in sn-2 position in lard.
Egg yolk	Lipids are dispersed in the form of lipoproteins, e.g. high density (HDL) and low density (LDL) lipoproteins. Lipoproteins from egg yolk are constituted by a hydrophobic core rich in TAG and cholesterol esters which is covered by a monolayer of phospholipids and apoproteins.
Milk lipids	Milk and dairy products are oil-in-water emulsions in which TAG are dispersed in an aqueous liquid phase (milk, cream), in a partially gelled phase (yoghurt, cheeses) or in a dry medium rich in proteins (powders). In cheese, fat is either dispersed as native milk fat globules (soft cheeses), (ii) present as fat globules more or less aggregated or coalesced, (iii) dispersed as small fat globules covered by proteins after high shear stress homogenisation (blue cheeses, some fresh cheeses), or (iv) in the form of free fat domains covered by milk polar lipids (hard-type cheeses). In butter, partially crystallised TAG forms the continuous phase, in which water droplets are dispersed, forming a water-in-oil emulsion. In whipped creams and ice-cream, TAG are present at the gas/water interface and participate in foam stabilisation.

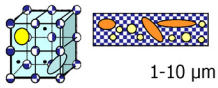
Organisation	Description
Oil seeds	Lipids are stored in organites called oleosomes, which are constituted by a hydrophobic core rich in TAG surrounded by a monolayer of phospholipids and proteins. The oleosins have strong steric hindrance. This organization ensures the oleosomes very high stability against thermal or detergents injuries.

Macroscopic scale

This level of complexity defines the lipid structure and lipid substructure in the food matrix (**Table 1.4**). The continuous lipid phase structure is present in foods with a high amount of fat in their composition. The free fat is a sub-structure consisting purely on bulk of fat with no other nutrient components interacting with it. Other examples include the butter, in which some hydrophilic components are also present in the form of water-in-oil emulsions. Lipids can be also present in a continuous aqueous phase structure, as the case of the dairy products and the egg yolk. The solid structure is the most complex. In these foods, fats are trapped by structural elements formed by other nutrients such as protein or carbohydrates, as in the case of meat or bread.

Table 1.4. Lipids classification according to their structure within the food matrix according to Michalski et al. (2013) [38]

Lipid structure	Lipid sub-structure	Graphical representation	Foods included
Continuous lipid phase	• Free fat • Water-in-oil emulsion • Particles in solid fat		• Olive oil • Butter • Chocolate
Continuous aqueous phase	• Oil-in-water emulsion • Intracellular lipid droplets and membrane structures		• Milk and dairy products • Egg yolk

Lipid structure	Lipid sub-structure	Graphical representation	Foods included
Complex solid structure	• Lipid inclusion in a protein and CH matrix • Lipid inclusion in a protein matrix		• Bread • Meat

1.6. FOOD DIGESTION

Digestion of foods is a mechanical and enzymatic process that occurs along the gastrointestinal tract allowing for the breakdown of nutrients into their forming components, so they can be absorbed and utilised by the organism. During the process of digestion, the first step for a nutrient to be bioaccessible is its physicochemical release from the food matrix and its conversion to a molecular form that is able to be absorbed by the intestine cells. The bioaccessibility of a nutrient will depend on several factors: the structure of the food matrix and the presence of other food components (intrinsic factors), and the conditions of the gastrointestinal environment and the presence of enzymatic enzymes (extrinsic factors) [47]. Therefore, digestion can be considered an extremely complex process, in which interactions between food properties and individual characteristics play a crucial role in nutrient digestion and absorption fate. Food digestion occurs in three steps, oral, gastric and intestinal.

Oral digestion

Food digestion starts in the mouth. Mechanical force of mastication is the first and the main contributor to food matrix disruption in which it is broken down into smaller pieces. At this initial stage the enzymatic action is also started, mainly by the α-amylase contained in the saliva, which starts the breakdown of carbohydrate polysaccharide chains. The environmental pH in the mouth is around 7. The resulting matter of the oral digestion is called bolus, which is swallowed and through the esophagus it gets to the stomach [48] (**Figure 1.6**).

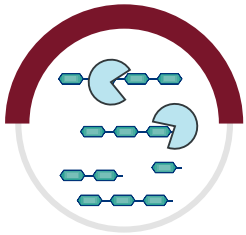


Figure 1.6. Enzymatic action during oral digestion. α-amylase breaks down polysaccharide chains

Gastric digestion

When the bolus reaches the stomach in the empty stage, it produces a buffering effect in the environmental pH, which increases from 1-2 to around 5, depending on the nature of the food. Then the gastric acidic secretion produced by the proton pumps makes the pH decrease to values close to 3, which are maintained along the gastric digestion [49]. Enzymes contained in the gastric juice include mainly trypsin, which is mainly responsible of proteolysis so proteins are broken down into peptide packages [50]; and also gastric lipase, which is the agent starting triglyceride digestion with affinity to the sn-3 position of the TAG molecule, reaching lipolysis extents up to 10-30% of total lipids [51]. When the bolus is mixed with the gastric fluids it forms the chyme. The mechanical agitation of the stomach walls contributes to the further breakdown of the bolus, and when the resulting parts achieve a small enough size, they pass the pylorus, a constriction muscle at the lower end of the stomach that directly opens into the duodenum (**Figure 1.7**).

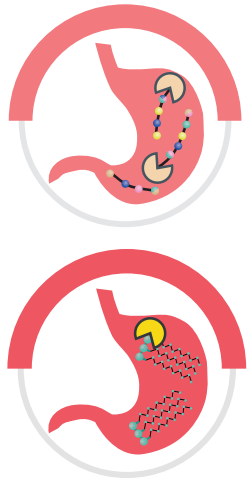


Figure 1.7. Enzymatic action during gastric digestion. Tripsin attacks protein molecules resulting in the release of peptides (up). Gastric lipase starts triglycerides digestion (down)

Intestinal digestion

When the chyme enters the duodenum, the first part of the small intestine, the pancreatic juice and the bicarbonate secretion produced by the pancreas start the intestinal digestion [50]. The pH increases from around 3 to up to 7, which is the optimal for pancreatic lipase activity [51]. The enzymatic activity at this stage of digestion includes the action of the amylases and proteases, which continue the breakdown of carbohydrates and proteins respectively, monosaccharides and aminoacids resulting as products.

The breakdown of triglycerides (fat) into their forming components, i.e. free fatty acids, supposes a more complex process, especially in the case of CF, in which lipases come from the enzymatic supplement, and the environmental conditions in the lumen, e.g. decreased pH, hinder their activity [52].

First, lipids need to be accessible to digestive enzymes so that their digestion and absorption can occur. The accessibility of TAG to lipases can be hindered by the characteristics of the food matrix, including their composition, supramolecular structure and mechanical behaviour. At this point of the digestion process, the food matrix has already been disrupted by mastication and gastric agitation, diluted by the digestive fluids, and their nutrients have been partially or totally digested by enzymes in the mouth and the stomach. Depending on food matrix properties, the rate and extent of lipids digestion will be conditioned as later on explained (**section 1.7**).

Pancreatic lipase is highly specific for the sn-1 and sn-3 positions of the triacylglycerol molecule, so the breakdown results into the release of 2 free fatty acids and 1 monoacylglycerol, formed by the glycerol backbone plus 1 fatty acid attached to the sn-2 position [41]. Pancreatic lipase may also continue the digestion of partially hydrolysed lipids at the gastric stage, i.e. diacylglycerols, by removing the fatty acid attached to the sn-3 position. As lipases release free fatty acids, bile salts, among other agents, contribute to remove these resulting products from the surface of the fat globule in order to facilitate the action of the lipases [53].

Once the TAG molecule is hydrolysed, the resulting products have to be absorbed by the intestine wall cells. For it, lipolytic products have to be solubilised, by for example as bile salt micelles and phospholipid vesicles, which are transited to the epithelial cells for absorption [53].

Along the small intestine, final products of digestion of carbohydrates and protein are absorbed too (i.e. monosaccharides and amionacids). The next stage in digestion is the large intestine, where mainly reabsorption of water occurs, with no further nutrient digestion or absorption by enzymatic activity (**Figure 1.8**).

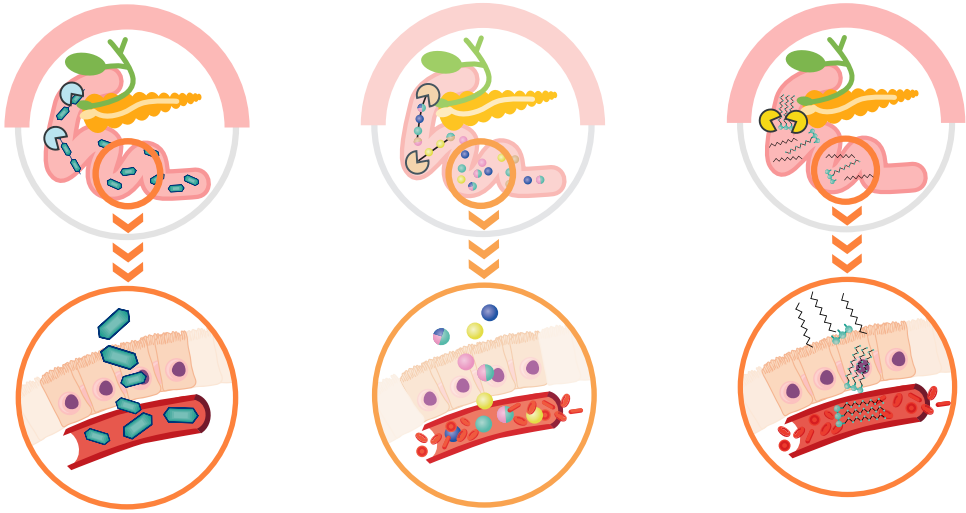


Figure 1.8. Enzymatic action during gastric digestion. Tripsin attacks protein molecules resulting in the release of peptides (up). Gastric lipase starts triglycerides digestion (down)

1.7. FACTORS AFFECTING LIPID DIGESTION

As previously explained, there is scarce information about the role of the factors affecting lipid digestion of foods under the gastrointestinal conditions of CF. Besides, little is known about the digestion of real foods (understood as real food matrices), but rather studies have focused on monocomponent systems or ideal lipid emulsions [37]. In this section, the most updated evidence about the influence of food components and gastrointestinal environment on lipid digestion is provided.

FOOD INTRINSIC FACTORS

Dietary lipids need to be accessible to digestive enzymes so that their digestion and absorption can occur. The accessibility of TAG to lipases can be hindered by the characteristics of the food matrix, including their composition, supramolecular structure and mechanical behaviour. The food matrix, which can be composed of proteins, sugars, starch and fibres, is destroyed during mastication, diluted and dissolved by saliva and gastric juice and hydrolysed by the digestive enzymes, which

allows the release of the embedded lipids and/or the access of the lipases to their substrates [53]. This is why both the composition and the structure of food matrix affect the digestion fate of dietary lipids.

Influence of triglyceride composition and structure on lipolysis

Fatty acid chain length and degree of unsaturation along with their stereospecific positional distribution may impact TAG rate and extent of lipolysis [54].

The rate and extent of TAG digestion during *in vitro* digestion decreases with increasing fatty acid chain length in this order: short-chain triacylglycerol (SCT) > medium-chain triacylglycerol (MCT) > long-chain triacylglycerol (LCT). This is ascribed to the fact that FFAs released from LCTs can accumulate at oil droplet surfaces, thereby restricting easy lipase access to other TAGs [55,56]. By contrast, medium or short-chain FFAs have a higher affinity for water and rapidly move into the surrounding aqueous phase after formation, making it easier for the lipase to hydrolyse as-yet unaffected TAGs [57]. However, this effect of long-chain FFAs delaying lipid digestion is dependent on bile concentrations as higher bile salt concentrations result in a higher rate and extent of LCT digestion [58].

The positional distribution of fatty acids within TAGs (especially long-chain fatty acids at the sn-1 or sn-3 positions) affected lipid digestion. Fatty acids released from the sn-1 and sn-3 positions often have different metabolic fates than fatty acids retained in the sn-2 position [41]. These metabolic fates depend on the fatty acid chain-length and stereospecific location on the triglyceride. Short and medium chain fatty acids (≤10 carbon atoms) can be solubilized in the aqueous phase of the intestinal contents, where they are absorbed. Longer-chain fatty acids, such as palmitic and stearic, have low coefficients of absorption because they have to first form micelles to be absorbed.

The degree of unsaturation of TAGs (i.e., poly vs. mono-unsaturation) does not appear to significantly affect lipid digestion, although work remains to be done in this area as scarce reports are available [37].

Influence of food matrix components on lipolysis

In foods with oil-in-water emulsion lipid structure, nominally, a protein film present at an oil-water interface will be easily broken down by proteases and displaced by bile salts [59]. Protein properties generally have little effect on lipid digestion [60].

As compared to animal protein, plant proteins may have a greater preventing effect against lipolysis during digestion. For example, wheat gliadins are much more insoluble and hydrophobic than diary proteins, so they form a more compact interfacial film that is resistant to displacement by bile salts. This hinders the diffusion of lipase towards the surface of the fat globules resulting in a lower extent of digestion. Another example is that soybean proteins are more resistant than β-lactoglobulin to interfacial displacement by bile salts at the oil-water interface, also leading to decreased lipid digestion [61,62].

Besides, when fat droplets are dispersed in a solid protein food matrix, like cheese or yoghurt, the structure of the surrounding food matrix becomes the dominant factor controlling digestion. During digestion, the 3D network protein structure within a food matrix can obstruct the diffusion of enzymes towards the surface of dispersed oil droplets. In these type of systems, lipolysis will be conditioned by the rate and extent of proteolysis: as the protein structure is broken down, the lipids are released from the food matrix, thus becoming accessible to lipases [63].

It has been reported that polysaccharide chains protect the oil-in-water emulsions against lipolysis by forming a thick hydrated layer that impedes lipases physical contact with the fat globule [64]. In fact, polysaccharides are used to thicken foods in industrial processing, as they have little propensity towards emulsification, given their lack of surface activity [65]. Overall, carbohydrates have been pointed as components decreasing lipolysis by other mechanisms, such as increasing viscosity of the digestion medium [66], wehat physically impedes the contact of lipases with the enzymes.

Influence of the lipid structure in the food matrix

Despite the scarce literature available on lipolysis in real foods, there are some studies with sound conclusions about the influence of the lipid structure in the food matrix, i.e. in almonds and in cheese. Guo et al. (2017) [37] compiled these papers, which are summarised below.

In almonds, oil bodies, which are the oil-bearing structures that may represent over 50 % of the nut weight, are located within thin-walled cells. So, the release of oil bodies from almond cells or the diffusion of lipase towards these cells is necessary for lipolysis. Approximately 10% of lipids in raw and roasted almonds have been observed to be released from almonds upon mastication with the cells remaining largely intact. The rate and extent of lipid digestion of almond cells were much lower than that of the isolated oil bodies (22 vs. 69% hydrolysis after 1 h digestion) during *in vitro* intestinal digestion [67]. *In vitro* digestion studies are highly consistent with in vivo human trials. Therefore, the digestion and bioaccessibility of lipids in almonds was regulated by the structure and properties of the cell walls surrounding the oil bodies.

Processed dairy products like cheese and yogurt are food emulsions with oil droplets incorporated within a solid or semi-solid matrix. Studies have shown that peptides and FFAs are more easily released from milks and yogurts (which are liquid and semi-solid matrices, respectively) vs. cheeses (which are solid matrices) owing to easier breakdown of the food matrix and greater accessibility by digestive enzymes in the former two. For cheese, the structural characteristics and hardness are determinants of lipid digestion. Fang et al. compared the disintegration and lipolysis of cheeses with different textural properties [68], and found that regular Cheddar cheese had a significantly higher extent of lipid digestion than light Mozzarella cheese. The larger fat globules in Cheddar cheese acted as weak points in its microstructure and texture, which led to a greater decrease in hardness and greater disintegration during *in vitro* digestion. By contrast, light Mozzarella consisted of a denser fibrous protein matrix due to the stretching step that occurs during its fabrication. Its protein matrix

showed a lower rate of disintegration and protein hydrolysis, thereby provid ing enhanced protection to dispersed fat and hence a lower extent of digestion [68].

EXTRINSIC FACTORS

Extrinsic factors in the process of digestion are those non-related to the food, but to the characteristics of the individual. Hormone alterations may change the action of key elements of the digestion process, such as transit time or of digestive fluids' secretion [69]. The altered physiological status, such as inflammation or infection, can also modify digestion and absorption of nutrients [70]. More evidently, the age of the individual is also determinant of this process. For example, new-borns do not have a completely developed digestive system, including a lack of secretion of digestive enzymes or the faster transit time [71]. In contrast, in elderly, several factors are affected, starting with the difficulties in mastication and the progressive decrease of enzymatic secretions, such as lactase [72]. Other specific pathologies do also imply complications in digestion, as it is the case of the short bowel syndrome, in which the nutrient absorption section of the intestine is drastically reduced [73].

In the particular case of CF, a series of alterations have a great impact on intestinal digestion. Pancreatic insufficiency is the main contributor to this alteration, since it impairs the secretion of pancreatic enzymes and bicarbonate to the duodenum. Other particular conditions in CF may include altered bile salts concentration and composition. These alterations in CF may lead to a wide range of possible sets of individual gastrointestinal conditions that would modify the action of PERT.

Gastric pH

In the stomach, pH is maintained at an average 3 during the gastric digestion [74]. However, it can be modified by the use of proton pump inhibitors (PPI) which block the secretion of protons making the gastric fluid less acidic. PPIs may be used in CF in order to palliate possible gastric reflux secondary to the disease. As a consequence of the inhibitory effect on acid secretion caused by PPI use, the environment at the stomach

can reach pH of 4 and 5. This increase may have an impact on proteolysis, as the proteases have an optimal pH around 3-4. Another effect of the use of PPIs would be that the chyme passes into the small intestine at a higher pH than 3, so despite decreased bicarbonate secretion, the pH increase at that stage would be higher, favouring the activity of pancreatic lipase [75].

Intestinal pH

Despite of the fact that pancreatic enzymes are taken to palliate the insufficiency, the lack of bicarbonate creates an acidic environment, at which enzymes do not have an optimal activity. Some studies have proved that intestinal pH in CF oscillates between 4.5 and 6.5, while in healthy individuals it reaches values higher than 7 [7,8, 76-78]. In addition, the time required to increase pH of the gastric content entering the duodenum is around 1 minute in healthy conditions whilst in CF it is delayed up to 30 minutes [76]. The overall lower pH at this stage unequivocally reduces the enzymatic activity, thus the PERT efficacy.

Bile salts concentration

Bile salts are bio-surfactant compounds with amphiphilic behaviour which play a crucial role in lipid digestion and absorption. During lipid digestion, they adsorb onto fat droplets and remove other materials such as proteins, emulsifiers and lipolysis products from the lipid surface, this way facilitating the access of lipases. To enable lipolysis products absorption, these compounds contribute to the formation of aggregates able to solubilise and transport lipid soluble compounds (such as free fatty acids and monoglycerides) through the gut cells where they can be absorbed [79].

Bile salts concentration could be up to 10 times lower in the case of CF and thus the 1mM condition was considered in our study [80]. Studies conducted several years ago, aimed at supplementing CF patients with taurine to achieve better digestion of fat. Literature, however, gathers controversial conclusions on this topic, some studies pointing a non-effect outcome [81,82] while others confirming its beneficial role as

adjuvants [83,84]. The discrepancy may be related to different experimental designs and assessed outcomes.

Summary of factors affecting digestion in Cystic Fibrosis

Table 1.5. Summary of factors affecting digestion in Cystic Fibrosis

Intrinsic factors	Extrinsic factors
Food properties	Individual characteristics
• Triacylglycerol composition • Food matrix components • Lipid organisation in the food matrix • Interactions between nutrients	• Gastric pH • Intestinal pH • Bile salts concentration • Volume of digestive fluids • Enzymes concentration

1.8. *IN VITRO* DIGESTION MODELS FOR FOOD AND HEALTH RESEARCH

When aiming to assess food digestion, available methodologies can be classified into four groups: animal studies, human studies, cell cultures, *in vitro* studies and in silico studies. In vivo feeding methods, using animals or humans, usually provide the most accurate results, but they are time consuming and costly, which is why much effort has been devoted to the development of *in vitro* procedures [85]. *In vitro* digestions methods simulate physiological digestion processes in lab, and they are widely used to study the gastrointestinal behaviour of food or pharmaceuticals.

Advantages of *in vitro* digestion methods

Compared to human in vivo studies, *in vitro* methods have the advantage of being rapider, less expensive, and have no ethical restrictions. Besides, they allow for a large number of samples to be measured in parallel for screening purposes.

Reproducibility, choice of controlled and reproducible conditions and easy sampling at the site of interest make *in vitro* models very suitable for addressing the study of food digestion. Among other factors, *in vitro* digestion methods can mimic the physiological in vivo digestion by taking into account digestive enzymes, pH, digestion time and salts concentration of the digestive fluids [24].

This way, it is possible to know the status of the digestion reactions at every specific point of the process, and to attribute the results only to the analysis conditions. In contrast, in vivo studies only allow for the evaluation of digestion at certain points, mainly at the end (e.g. measuring levels of a nutrient in plasma or faeces analysis, once digestion is finished), with no possibility to monitor the rest of the process. In this sense, in vivo studies would be indicated only after the influence of the determining study factors of digestion have been analysed *in vitro* [86].

Application of *in vitro* digestion methods

The application of this methodology can address such diverse scientific questions, like the digestibility and bioaccessibility (i.e. the amount of a compound that is released from the matrix and is considered to be available for absorption through the gut wall) of pharmaceuticals, mycotoxins, and macronutrients such as proteins, carbohydrates and lipids. They have also been used to study matrix release of micronutrients such as minerals and trace elements, and secondary plant compounds including carotenoids and polyphenols [24].

A harmonised international protocol for *in vitro* digestion of foods

In the light of the high application potential of *in vitro* methods, Minekus et al. (2014) published the harmonised international protocol to conduct this type of studies [37]. This protocol describes a “smallest common denominator”, i.e. a set of conditions that are close to the physiological situation, are practical, and can be seen as a basic suggestion to address various research questions. Authors strongly highlight that further amendments of their suggested conditions may be needed, for example to

simulate digestion in infants or the elderly, or in our case, in CF. **Table 1.6** summarises the basic digestion conditions described by this international protocol.

Table 1.6. Summary of the digestion conditions to conduct an *in vitro* digestion study according to the harmonised standard protocol proposed by Minekus et al. (2014) [24]

Parameter	Oral step	Gastric step	Intestinal step
pH	7	3	7
Duration (min)	2	120	120
Volumetric proportion (total volume)	Food:SSF 1:1 (10 ml)	Bolus:SGF 1:1 (20 ml)	Chyme:SIF 1:1 (40 ml)
Enzymes	Salivary amylase (75 U/ml)	Pepsin (2000 U/ml)	Pancreatin (Pancreatic lipase 2000 LU/ml)
Bile salts concentration	-	-	10 mM

Complementary analysis derived from *in vitro* digestion

During the process of *in vitro* digestion there is a wide range of complementary analysis that can be applied according to the objective of the investigation. In the study of macronutrients hydrolysis, such as lipids, there are a series of measurements that can support the follow-up of the process at different points (**Table 1.7**).

Table 1.7. Analytical determinations attached to the process of *in vitro* digestion to monitor and assess lipolysis

Complementary analysis	Digestion point	Description
Texture of the food sample	Before digestion	The food sample is submitted to a TPA analysis and textural properties such as hardness or adhesiveness are determined
Viscosity of the digestive content	During/after gastric stage	The entire digestion medium is submitted to a viscosity analysis at a certain point of the gastric digestion, with for example the Bostwick consistometre. During the intestinal stage viscosity changes are not detectable.
Matrix degradation index	During/after intestinal stage	It expresses the relationship between the initial weight of the food sample and the solid phase of the digesta after separation from the micellar phase.
Total free fatty acids released	During/after intestinal stage	A common technique to quantify total free fatty acids which consists of a specific colorimetric assay which is based on a spectrophotometry measurement.
Free fatty acids profile	During/after intestinal stage	The micellar phase of the digesta is first submitted to esterification of the free fatty acids, and then a gas chromatography – mass spectrometry. Besides the identification of the released free fatty acids, quantification can be obtained by means of referencing the areas to the specific fatty acids calibration curves.

In vitro digestion studies of different foods might offer a useful tool to shed light on the understanding of lipolysis in CF-specific conditions and to give guidance on PERT dosing. Nevertheless, up to now, there are only a few known studies on lipid digestion in real or complex foods, that limits the translation of knowledge from *in vitro* digestion outcomes to the clinical practice.

REFERENCES

1. Farrell, P. M. (2008). The prevalence of cystic fibrosis in the European Union. *Journal of Cystic Fibrosis*, *7*(5), 450-453.
2. Zolin, A., van Rens, J., Fox, A., Iansa, P., Preftitsi, A., Pypops, U., ... & Vebert Olesen, H. (2014). ECFS Patient Registry, annual report 2010. *European Cystic Fibrosis Society, Karup, Denmark*.
3. Gaskin, K. J. (2013). Nutritional care in children with cystic fibrosis: are our patients becoming better?. *European journal of clinical nutrition*, *67*(5), 558.
4. O'Sullivan, B.P., Freedman, S.D. (2009) Cystic fibrosis. *Lancet*, 373:1891– 904.
5. Sikkens, E. C., Cahen, D. L., Kuipers, E. J., & Bruno, M. J. (2010). Pancreatic enzyme replacement therapy in chronic pancreatitis. *Best Practice & Research Clinical Gastroenterology*, *24*(3), 337-347.
6. Woestenenk, J. W., van der Ent, C. K., & Houwen, R. H. (2015). Pancreatic enzyme replacement therapy and coefficient of fat absorption in children and adolescents with cystic fibrosis. *Journal of pediatric gastroenterology and nutrition*, *61*(3), 355-360.
7. Clarke, L. L., Stien, X., & Walker, N. M. (2001). Intestinal bicarbonate secretion in cystic fibrosis mice. *Jop*, *2*(4 suppl), 263-267.
8. Rowe, S. M., Heltshe, S. L., Gonska, T., Donaldson, S. H., Borowitz, D., Gelfond, D., ... & Joseloff, E. (2014). Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. *American journal of respiratory and critical care medicine*, *190*(2), 175-184.
9. Stephenson, A. L., Mannik, L. A., Walsh, S., Brotherwood, M., Robert, R., Darling, P. B., ... & Stanojevic, S. (2013). Longitudinal trends in nutritional status and the relation between lung function and BMI in cystic fibrosis: a population-based cohort study–. *The American journal of clinical nutrition*, *97*(4), 872-877.
10. Efrati, O., Nir, J., Fraser, D., Cohen-Cymberknoh, M., Shosevov, D., … & Rivlin, J. (2012). Meconium ileus in patients with cystic fibrosis is not a risk factor for clinical deterioration and survival: the Israeli Multicenter Study. *Journal of pediatric gastroenterology and nutrition*, 50(2):173-178
11. Yen, E. H., Quinton, H., & Borowitz, D. (2013). Better nutritional status in early childhood is associated with improved clinical outcomes and survival in patients with cystic fibrosis. *The Journal of pediatrics*, *162*(3), 530-535.
12. Amin, R., & Ratjen, F. (2014). Emerging drugs for cystic fibrosis. *Expert opinion on emerging drugs*, *19*(1), 143-155.
13. Turck, D., Braegger, C. P., Colombo, C., Declercq, D., Morton, A., Pancheva, R., ... & Schneider, S. M. (2016). ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clinical Nutrition*, *35*(3), 557-577.
14. Borowitz, D., Baker, R.D., Stallings, V (2002). Consensus report on nutrition for pediatric patients with cystic fibrosis. *Journal of Pediatric Gastroenterology and Nutrition*, 35, 246-259.
15. Sinaasappel, M., Stern, M., Littlewood, J, et al. (2002). Nutrition in patients with cystic fibrosis: a European Consensus. *Journal of Cystic Fibrosis,* 1, 51-75.
16. Fieker, A., Philpott, J., & Armand, M. (2011). Enzyme replacement therapy for pancreatic insufficiency: present and future. *Clinical and experimental gastroenterology*, *4*, 55.
17. Woestenenk, W., van der Ent, C.K., Houwen, R.H. (2015). Pancreatic Enzyme Replacement Therapy and Coefficient of Fat Absorption in Children and Adolescents With Cystic Fibrosis. *Journal of Pediatric Gastroenterology and Nutrition,* 61: 355–360.
18. Riegler, G. & Expósito, I. (2001). Bristol scale stool form. A still valid help in medical practice and clinical research. *Tech. Coloproctol,* 5, 163–164.

19. Kalnins, D., Corey, M., Ellis, L., et al. (2005). Combining unprotected pancreatic enzymes with pH-sensitive enteric-coated microspheres does not improve nutrient digestion in patients with cystic fibrosis. *Journal of Pediatrics*, 146, 489 – 93.
20. Kalnins, D., Ellis, L., Corey, M., et al. (2006). Enteric-coated pancreatic enzyme with bicarbonate is equal to standard enteric-coated enzyme in treating malabsorption in cystic fibrosis. *Journal of Pediatric Gastroenterology and Nutrition*, 42,256 – 61.
21. Durie, P., Kalnins, D., Ellis, L.U. sesandabuses of enzyme therapy in cystic fibrosis. J R Soc Med 1998;91 (suppl 34):2–13.
22. Li Y, Hu M, McClements DJ. (2011) Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: Proposal for a standardised pH-stat method. Food Chem 126:498–505.
23. Hunter EJ. (2001) Studies on effects of dietary fatty acids as related to their position on triglycerides. Lipids 36:655–68.
24. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. (2014) A standardised static *in vitro* digestion method suitable for food – an international consensus. Food Funct Food Funct 5:1113–24.
25. Fang X, Rioux L, Labrie S, Turgeon SL (2016) Disintegration and nutrients release from cheese with different textural properties during *in vitro* digestion. FRIN 88:276-283
26. Bläckberg L, Hernell O, Bengtsson G, Olivecrona T (1979) Colipase enhances hydrolysis of dietary triglycerides in the absence of bile salts. J Clin Invest ;64:1303–8.
27. Andree M, Weber MD, Claude C, Roy MD, Claude L, Morin MD, et al. (1996) Malabsorption of bile acids in children with cystic fibrosis. N Engl J Med. 289:1001–5.
28. Hu M, Li Y, Decker EA, McClements DJ (2010) Role of calcium and calcium-binding agents on the lipase digestibility of emulsified lipids using an *in vitro* digestion model. Food Hydrocoll 24:719–25.
29. Messick J. A 21st-century approach to cystic fibrosis: optimizing outcomes across the disease spectrum. *J Pediatr Gastroenterol Nutr*. 2010;51(September):S1-7.
30. Eising JB, van der Ent CK, Teske AJ, Vanderschuren MM, Uiterwaal CSPM, Meijboom FJ. Young patients with cystic fibrosis demonstrate subtle alterations of the cardiovascular system. *J Cyst Fibros*. 2018:1-7.
31. Keogh RH, Szczesniak R, Taylor-Robinson D, Bilton D, Keogh RH. Up-to-date and projected estimates of survival for people with cystic fibrosis using baseline characteristics: A longitudinal study using UK patient registry data ☆. *J Cyst Fibros*. 2017.
32. Sutherland RE, Collins C, Brunner R, Ooi CY, Katz TE. 316 An historical perspective of dietary intake studies in children with CF. *J Cyst Fibros*. 2017.
33. Ishimo M-C, Belson L, Ziai S, et al. Hypertriglyceridemia is associated with insulin levels in adult cystic fibrosis patients. *J Cyst Fibros*. 2013.
34. Sutherland, R. E., Collins, C., Brunner, R., Ooi, C. Y. & Katz, T. E. (2017) 316 An historical perspective of dietary intake studies in children with CF. J. Cyst. Fibros. 16:S143.
35. Lairon, D. (2009). Digestion and absorption of lipids. In *Designing functional foods* (pp. 68-93).
36. Small, D. M. (1986). The physical chemistry of lipids. *Handbook of lipid research*, *4*, 285-343.
37. Guo, Q., Ye, A., Bellissimo, N., Singh, H., & Rousseau, D. (2017). Modulating fat digestion through food structure design. *Progress in lipid research*, *68*, 109-118.
38. Michalski, M. C., Genot, C., Gayet, C., Lopez, C., Fine, F., Joffre, F., ... & steering committee of RMT LISTRAL. (2013). Multiscale structures of lipids in foods as parameters affecting fatty acid bioavailability and lipid metabolism. *Progress in Lipid Research*, *52*(4), 354-373.
39. F.A. Norris, K.F. Mattil, A new approach to the glyceride structure of natural fats, J. Am. Oil Chem. Soc. 24(8) (1947) 274-275.

40. T.P. Hilditch, P.N. Williams, The chemical constitution of natural fats, 4 ed., Springer US, New York, 1964.
41. Hunter, J. E. (2001). Studies on effects of dietary fatty acids as related to their position on triglycerides. *Lipids*, *36*(7), 655-668.
42. J. Ubbink, A. Burbidge, R. Mezzenga, Food structure and functionality: a soft matter perspective, Soft Matter 4(8) (2008) 1569-1581.
43. K. Nyemb, C. Guérin-Dubiard, D. Dupont, J. Jardin, S.M. Rutherfurd, F. Nau, The extent of ovalbumin *in vitro* digestion and the nature of generated peptides are modulated by the morphology of protein aggregates, Food Chem. 157(0) (2014) 429-438.
44. K. Nyemb, C. Guérin-Dubiard, S. Pézennec, J. Jardin, V. Briard-Bion, C. Cauty, S.M. Rutherfurd, D. Dupont, F. Nau, The structural properties of egg white gels impact the extent of in vitro protein digestion and the nature of peptides generated, Food Hydrocolloids 54, Part B (2016) 315-327.
45. Parada, J., & Aguilera, J. M. (2007). Food microstructure affects the bioavailability of several nutrients. *Journal of food science*, *72*(2).
46. D.J. McClements, Food emulsions: principles, practices, and techniques, CRC press, New York, 2015.
47. Yen, E. H., Ms, H. Q. & Borowitz, D. (2013) Better Nutritional Status in Early Childhood Is Associated with Improved Clinical Outcomes and Survival in Patients with Cystic Fibrosis. J. Pediatr. 162, 530–535.e1.
48. Pedersen, A. M., Bardow, A., Jensen, S. B., & Nauntofte, B. (2002). Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. *Oral diseases*, *8*(3), 117-129.
49. Dressman, J. B., Berardi, R. R., Dermentzoglou, L. C., Russell, T. L., Schmaltz, S. P., Barnett, J. L., & Jarvenpaa, K. M. (1990). Upper gastrointestinal (GI) pH in young, healthy men and women. *Pharmaceutical research*, *7*(7), 756-761.
50. Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B., & Reppas, C. (2006). Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharmaceutical research*, *23*(1), 165-176.
51. Carrière, F., Renou, C., Lopez, V., De Caro, J., Ferrato, F., Lengsfeld, H., ... & Verger, R. (2000). The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test meals. *Gastroenterology*, *119*(4), 949-960.
52. Gregory, P. C. (1996). Gastrointestinal pH, motility/transit and permeability in cystic fibrosis. *Journal of pediatric gastroenterology and nutrition*, *23*(5), 513-523.
53. H. Mu, C.-E. Høy, The digestion of dietary triacylglycerols, Prog. Lipid Res. 43(2) (2004) 105-133.
54. E. Rogalska, S. Ransac, R. Verger, Stereoselectivity of lipases. II. Stereoselective hydrolysis of triglycerides by gastric and pancreatic lipases, J. Biol. Chem. 265(33) (1990) 20271-6.
55. R. Devraj, H.D. Williams, D.B. Warren, A. Mullertz, C.J.H. Porter, C.W. Pouton, In vitro digestion testing of lipid-based delivery systems: Calcium ions combine with fatty acids liberated from triglyceride rich lipid solutions to form soaps and reduce the solubilization capacity of colloidal digestion products, Int. J. Pharm. 441(1–2) (2013) 323-333.
56. J.P.R. Day, G. Rago, K.F. Domke, K.P. Velikov, M. Bonn, Label-free imaging of lipophilic bioactive molecules during lipid digestion by multiplex coherent anti-Stokes Raman scattering microspectroscopy, J. Am. Chem. Soc. 132(24) (2010) 8433-8439.
57. [L. Sek, C.J.H. Porter, A.M. Kaukonen, W.N. Charman, Evaluation of the in-vitro digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products, J. Pharm. Pharmacol. 54(1) (2002) 29-41.
58. R. Devraj, H.D. Williams, D.B. Warren, A. Mullertz, C.J.H. Porter, C.W. Pouton, In vitro digestion testing of lipid-based delivery systems: Calcium ions combine with fatty acids liberated from

triglyceride rich lipid solutions to form soaps and reduce the solubilization capacity of colloidal digestion products, Int. J. Pharm. 441(1–2) (2013) 323-333.

59. J. Maldonado-Valderrama, N.C. Woodward, A.P. Gunning, M.J. Ridout, F.A. Husband, A.R. Mackie, V.J. Morris, P.J. Wilde, Interfacial characterization of β-lactoglobulin networks: displacement by bile salts, Langmuir 24(13) (2008) 6759-6767.
60. H. Singh, A. Ye, Structural and biochemical factors affecting the digestion of protein- stabilized emulsions, Curr. Opin. Colloid Interface Sci. 18(4) (2013) 360-370.
61. C. Qiu, W. Sun, C. Cui, M. Zhao, Effect of citric acid deamidation on in vitro digestibility and antioxidant properties of wheat gluten, Food Chem. 141(3) (2013) 2772-2778.
62. C. Qiu, M. Zhao, E.A. Decker, D.J. McClements, Influence of protein type on oxidation and digestibility of fish oil-in-water emulsions: Gliadin, caseinate, and whey protein, Food Chem. 175 (2015) 249-257.
63. E. Dickinson, Emulsion gels: The structuring of soft solids with protein-stabilized oil droplets, Food Hydrocolloids 28(1) (2012) 224-241.
64. A. Nakamura, R. Yoshida, H. Maeda, M. Corredig, Soy soluble polysaccharide stabilization at oil–water interfaces, Food Hydrocolloids 20(2) (2006) 277-283.
65. E. Dickinson, Hydrocolloids as emulsifiers and emulsion stabilizers, Food Hydrocolloids 23(6) (2009) 1473-1482.
66. Lairon, D., Play, B., & Jourdheuil-Rahmani, D. (2007). Digestible and indigestible carbohydrates: interactions with postprandial lipid metabolism. *The Journal of nutritional biochemistry*, *18*(4), 217-227.
67. Grundy, M. M., Carrière, F., Mackie, A. R., Gray, D. A., Butterworth, P. J., & Ellis, P. R. (2016). The role of plant cell wall encapsulation and porosity in regulating lipolysis during the digestion of almond seeds. *Food & function*, *7*(1), 69-78.
68. Fang, X., Rioux, L. E., Labrie, S., & Turgeon, S. L. (2016). Disintegration and nutrients release from cheese with different textural properties during in vitro digestion. *Food Research International*, *88*, 276-283.
69. Carswell, K. A., Vincent, R. P., Belgaumkar, A. P., Sherwood, R. A., Amiel, S. A., Patel, A. G., & Le Roux, C. W. (2014). The effect of bariatric surgery on intestinal absorption and transit time. *Obesity surgery*, *24*(5), 796-805.
70. Ferrari, M., Cuenca-Garcia, M., Valtuena, J., Moreno, L. A., Censi, L., Gonzalez-Gross, M., ... & Sjöström, M. (2015). Inflammation profile in overweight/obese adolescents in Europe: an analysis in relation to iron status. *European journal of clinical nutrition*, *69*(2), 247.
71. Mooij, M.G., de Koning, B.A.E., Huijsman, M.L., de Wildt, S.N. Ontogeny of oral drug absorption (2012). Expert Opin. Drug Metab. Toxicol.
72. Casellas, F., Aparici, A., Casaus, M., & Rodrıguez, P. (2013). Self-perceived lactose intolerance and lactose breath test in elderly. European Geriatric Medicine, 4, 372–375.
73. O'Keefe, S. J., Buchman, A. L., Fishbein, T. M., Jeejeebhoy, K. N., Jeppesen, P. B., & Shaffer, J. (2006). Short bowel syndrome and intestinal failure: consensus definitions and overview. *Clinical Gastroenterology and Hepatology*, *4*(1), 6-10.
74. Armand M, Hamosh M, Philpott JR, Resnik AK, Rosenstein BJ, Hamosh A, et al. (2004) Gastric Function in Children with Cystic Fibrosis: Effect of Diet on Gastric Lipase Levels and Fat Digestion. Pediatr Res. 55:457–65.

75. Zentler-Munro, P. L., Fine, D. R., Batten, J. C., & Northfield, T. C. (1985). Effect of cimetidine on enzyme inactivation, bile acid precipitation, and lipid solubilisation in pancreatic steatorrhoea due to cystic fibrosis. *Gut*, *26*(9), 892-901.
76. Gelfond D, Ma C, Semler J, Borowitz D (2013) Intestinal ph and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. Dig Dis Sci 58:2275–81.
77. Robinson PJ, Smith AL, Sly PD, Al RET (1990) Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. Dig Dis Sci 35:1299–304.
78. Balzola F, Cullen G, Ho GT, Russell R. Delayed small bowel transit in children with cystic fibrosis and pancreatic insufficiency: Commentary. Inflamm Bowel Dis Monit. 2014;14(2):65–6.
79. Norman AP. (1979) Intestinal bile salts in cystic fibrosis. Archives of disease in childhood 54:19-24
80. Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science*, *165*(1), 36-46.
81. Thompson G, Robb T, Davison G (1987) Taurine supplementation, fat absorption and growth in cystic fibrosis. J Pediatr Gastroenterol Nutr 111:501–6.
82. Merli M, Bertasi S, Servi R, Diamanti S, Martino F, De Santis A, et al. (1994) Effect of a Medium Dose of Ursodeoxycholic Acid with or without Taurine Supplementation on the Nutritional Status of Patients with Cystic Fibrosis: A randomized, placebo-controlled, crossover trial. J Pediatr Gastroenterol nutr 19:198–203.
83. Colombo C, Arlati S, Curcio L, Maiavacca R, Garatti M. (1988) Effect of Taurine Supplementation on Fat and Bile Acid Absorption in Patients with Cystic Fibrosis 143:151-156
84. Belli D, Levy E, Darling P, Leroy C, Lepage G, Giguère R, et al. (1986) Taurine Improves the Absorption in Patients With Cystic Fibrosis. Pediatrics 80;517-23.
85. Ménard, O.; Cattenoz, T.; Guillemin, H.; Souchon, I.; Deglaire, A.; Duponot, D.; Picque, D. 2014. Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry*, **145**: 1039-1045.

2. HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

Up to date there is not a scientifically valid method to adjust pancreatic enzyme replacement therapy (PERT) in Cystic Fibrosis. Food properties have proved to be determinants in lipid digestion. Thus, foods and dietary habits could have an impact on PERT efficacy, and ultimately on PERT dose. Therefore, the hypothesis of the present thesis is that a method to optimally adjust PERT dose could be based on foods characteristics, such as lipid structure and food matrix properties. In addition, the hypothesis poses that the method could be refined if adapted to patients' individual characteristics, such as age, type of mutation or nutritional status.

In order to assess this hypothesis a multi- and trans-disciplinary European Project (MyCyFAPP) was designed, involving nutrition, food science and technology, medicine and mHealth among other fields. The project was applied for and granted with the Horizon 2020 framework programme of the European Union. The Project description is reported in Chapter 1 (Paper 1).

The main goal of the project and of this thesis is to develop an evidence-based method to adjust PERT in Cystic Fibrosis. To achieve it, a step-wise approach was followed, including three stages of the research conducted, and defined as three research questions. Under the heading of each research question, specific objectives were posed.

Research Question 1: *How is the current situation with regard to PERT dose and nutrition in Cystic Fibrosis children in Europe as referred to the medical guidelines?*
To answer to this question, the following specific objectives were posed:

- To establish the nutrient and energy intake of paediatric patients in different European cystic fibrosis units

- To define their nutritional habits and dietary patterns
- To establish their nutritional status
- To determine the PERT dose criteria applied in different CF centres

These results are provided in Chapter 2, through papers 2, 3 and 4.

Research Question 2: *How do gastrointestinal conditions and food properties affect lipid digestion by PERT?*

Four specific objectives were established to address this question:

- To determine the influence of the gastrointestinal environment conditions on lipolysis kinetics and extent: gastric and intestinal pH, bile salts concentration and composition, volume of the digestive fluids and amount of fat in digestion medium.
- To determine the role of intestinal pH and bile salts concentration on lipolysis extent in the entire range of real foods
- To determine the role of nutrient composition and the lipid structure within the food matrix on lipolysis extent in the entire range of real foods
- To determine the influence of the food matrix properties, including nutrient composition, texture and viscosity conferred to the digestion medium, on lipid digestion of olive oil and butter as assessed by free fatty acid release profile

The Papers contained in Chapter 3 (5-7) report the results.

Research Question 3: *can PERT dose be optimally adjusted according to food properties and patients' characteristics?*

The following specific objectives were established to address this question:

- To define the theoretical optimal dose of the enzymatic supplement in lab, under simulated cystic fibrosis gastrointestinal conditions, by *in vitro* testing a range of doses of the enzymatic supplement for a selection of frequent consumed foods

- To define a model to predict the optimal dose of PERT according to food characteristics
- To design a pilot study to test the validity of the results of the theoretical optimal doses obtained *in vitro* in real patients with cystic fibrosis:
- To establish the relationship between the *in vitro* and the in vivo scenarios in terms of lipid digestion
- To establish the influence of the individual patients' characteristics on the efficacy of the *in vitro* method to adjust the dose of PERT
- To define a model to predict the optimal dose of PERT according to patients' individual characteristics

Paper 8 (*in vitro* part) and Papers 9 and 10 (in vivo study) complied in Chapter 4 report the results of this last part of the research.

3. EXPERIMENTAL PLAN

In order to accomplish the objectives, the experimental plan detailed in this part was carried out. All the research activities were conducted within the framework of MyCyFAPP Project as specific tasks of the project implementation. It is divided in three sections, according to the nature and the stage of the research. Furthermore, bibliography in relation to the topics was periodically reviewed. The experimental plan comprised the period between September 2014 and May 2018. The candidate conducted the research at two institutions: Universitat Politècnica de València and Instituto de Investigación Sanitaria La Fe. Additionally, part of the research was conducted at Gasthuisberg Hospital – Katholieke Universitetit Leuven.

3. EXPERIMENTAL PLAN

3.1. MULTICENTER STUDY TO ASSESS PERT AND NUTRITION IN PATIENTS WITH CYSTIC FIBROSIS

For the first stage of the research a multicentre European study on nutritional habits and PERT dose use was conducted (**Figure 3.1**). The study protocol was prepared and approved by the Ethics Committees of the 6 participating European CF units: Instituto de Investigación Sanitaria La Fe (Valencia, Spain), Hospital Universitario Ramón y Cajal (Madrid, Spain), Associação per a la Investigação e Desenvolvivmento da Facultade de Medicina (Lisbon, Portugal), Gasthuisberg Hospital, KU Leuven (Leuven, Belgium), Erasmus Medisch Centrum Sophia's Children Hospital (Rotterdam, The Netherlands) and Ospedale Maggiore Policlinico – Università degli studi di Milano (Milan, Italy). A specific food record was developed in order to collect the necessary information. Nutritional composition databases were adapted to the needs of the study, including the completion of the nutrient facts information and the classification of all the food items into food groups and subgroups according to a common consensus criterion. An online system was developed in order to collect all the data and to perform the automatic calculations that allowed to obtain the results in terms of: nutrient intake, food groups intake and PERT use in the study populations.

3.2. *IN VITRO* DIGESTION STUDIES

The second stage consisted of the *in vitro* digestion studies that were conducted at the FooDiHealth lab, at the Instututo de Ingeniería de Alimentos para el Desarrollo of the Universitat Politècnica de València. The harmonised international protocol to conduct *in vitro* digestion methods was used as the basis to develop a protocol adapted to the objectives of the research: studying the role of the gastrointestinal conditions, food properties and PERT dose on lipolysis (**Figure 3.2**). The work in the lab consisted of the preparation of the simulated digestive fluids and the food sample. Then the oral, gastric

and intestinal stages of digestion were either conducted by the pH-stat method or the method consisting of simulating digestion in falcon tubes agitated head-over-heels within a thermostated chamber. At different stages of the process, analytical techniques were applied: food sample texture measurement, viscosity measurement of the digestion medium, total free fatty acids released, and free fatty acids profile.

3.3. PILOT STUDY TO ASSESS AN EVIDENCE-BASED METHOD FOR PERT

The last part of the research consisted of the translation of the generated knowledge by means of the *in vitro* digestion studies to the clinical practice. A protocol was designed in order to test the efficacy of the *in vitro* theoretical optimal dose of PERT for a selection of foods in real patients (**Figure 3.3**). The study was approved by the ethics committee of the participating centres. Patients followed during 24h a fixed diet with a fixed PERT dose at the time they collected faeces in order to assess the achieved fat digestion of the test meal with the fixed PERT dose. To guarantee that faeces corresponded to the study period, they were marked with colorimetric dyes administrated at the pertinent times. The influence of the patients' individual characteristics on fat digestion achieved were studied with the aim of adapting the dosing criteria to these characteristics. The free fatty acids profile was also studied in order to assess the relationship between digested and absorbed fat.

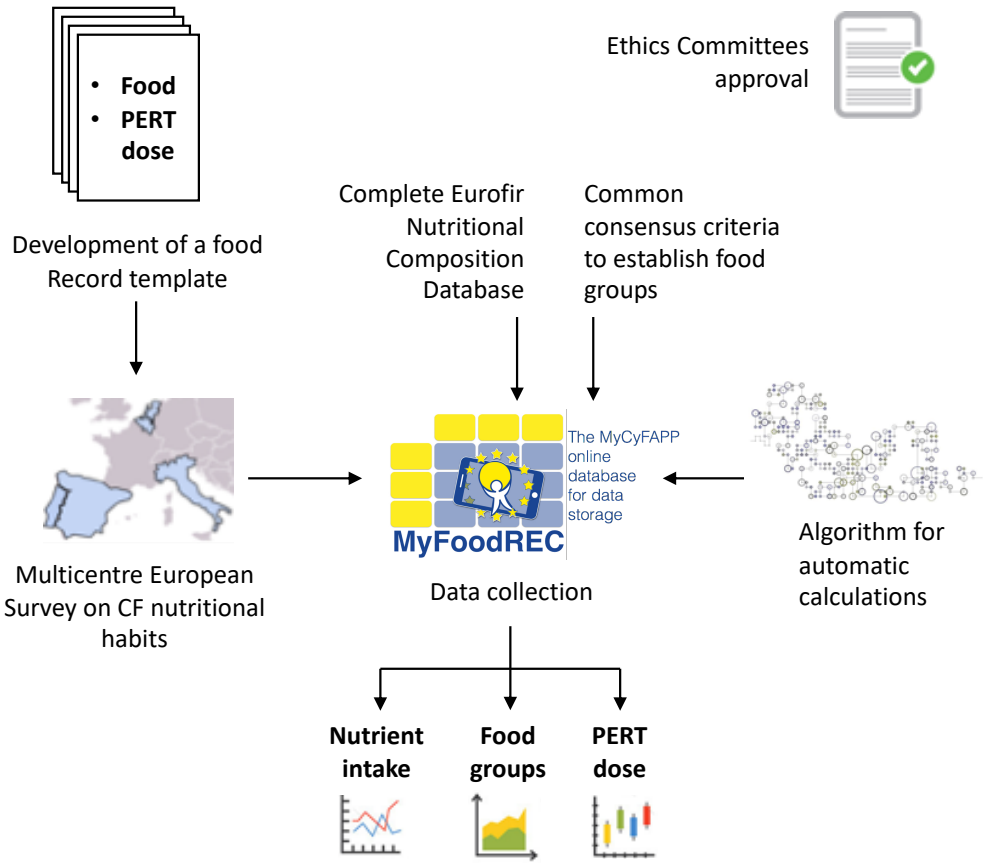


Figure 3.1. Diagram of the experimental plan applied to conduct the European multicentre study on nutritional habits and PERT use. This experimental plan allowed for the generation of the results reported in Chapter 2.

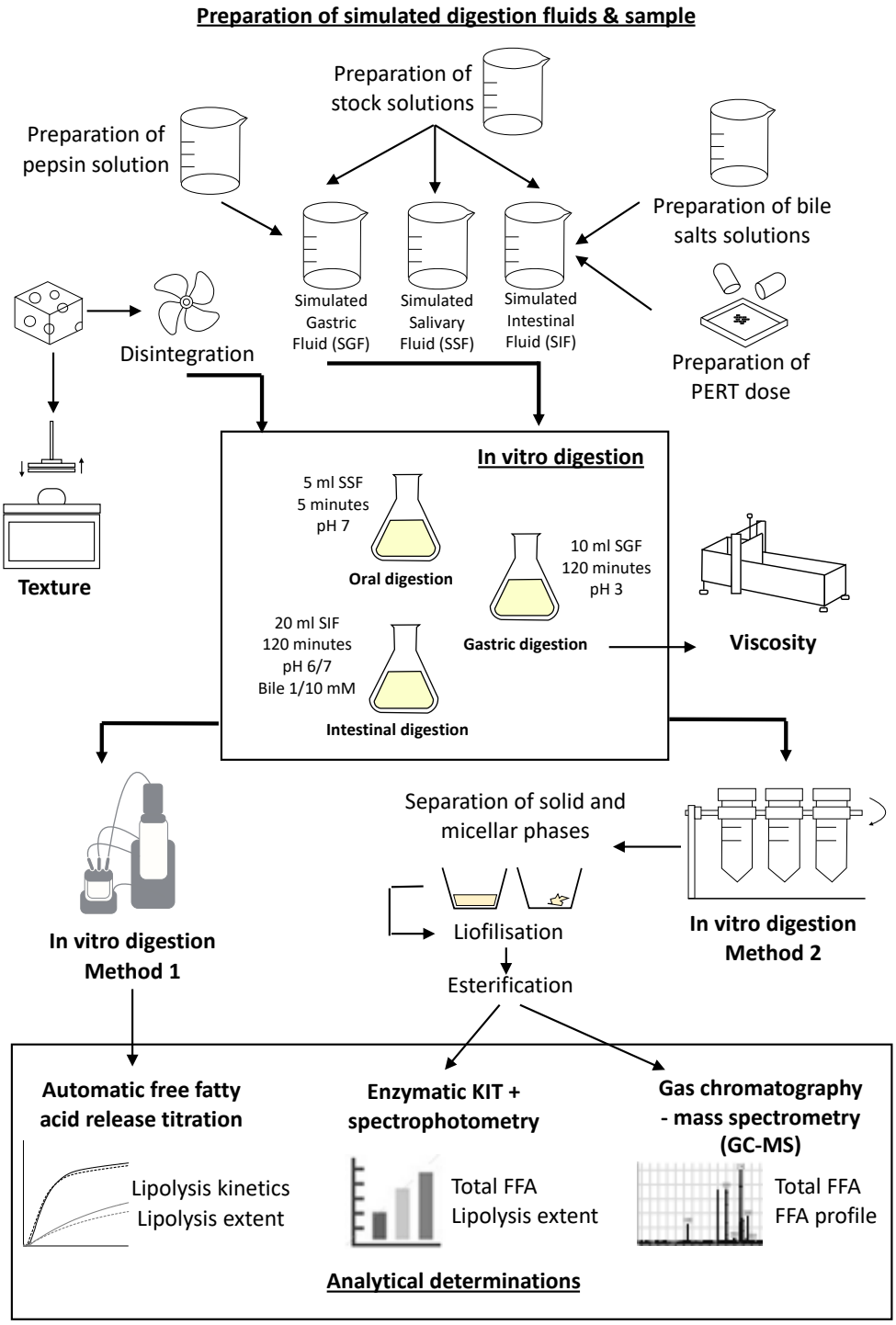


Figure 3.2. Diagram of the experimental plan applied to conduct the *in vitro* digestion studies. This experimental plan led to the results reported in Chapter 3.

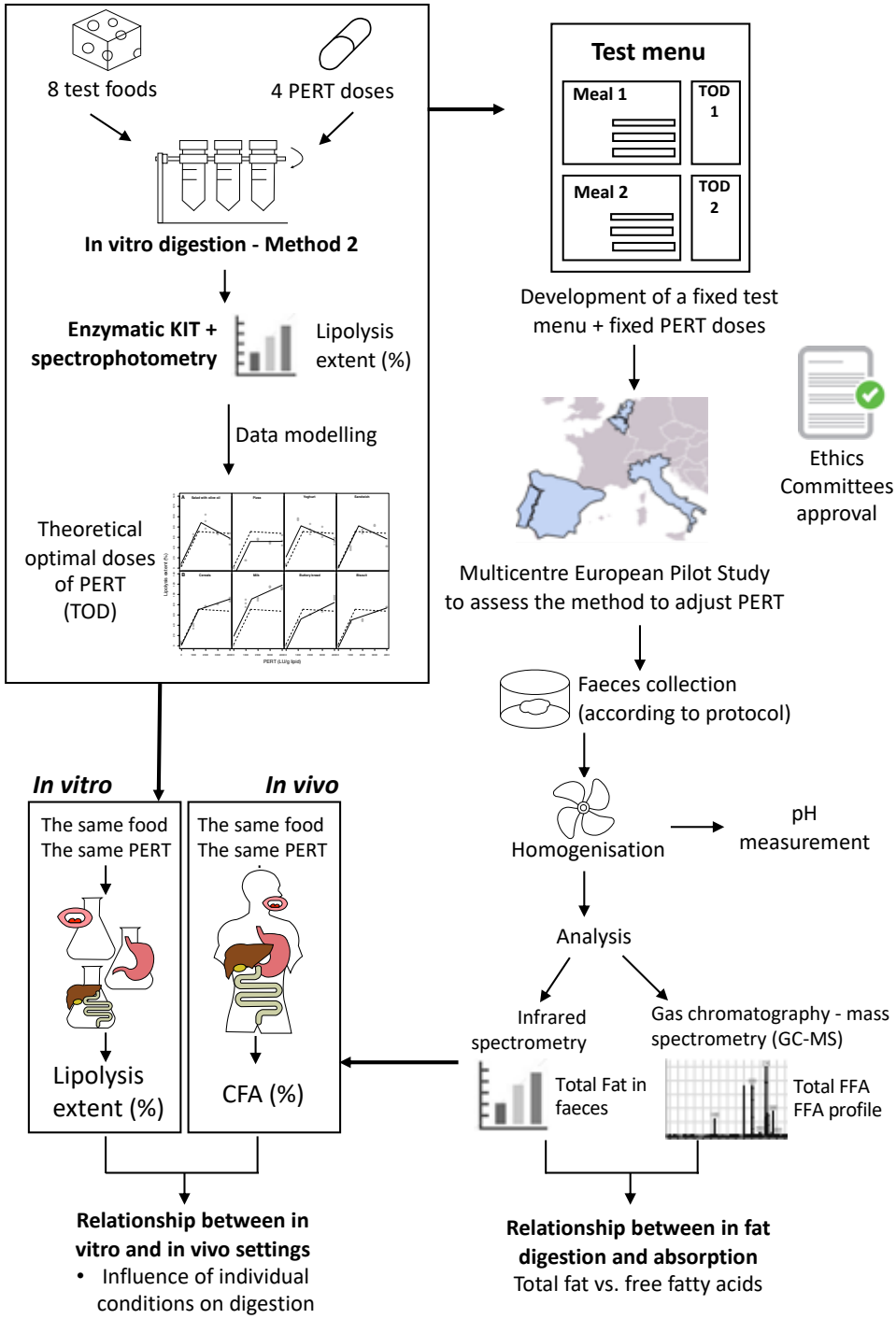


Figure 3.3. Diagram of the experimental plan applied to conduct the development and validation of the evidence-based method to adjust PERT. It led to the results explained in Chapter 4.

4. RESULTS

The publications resulting from the research performed in the scope of this PhD thesis are presented in this section. For each publication, the PhD candidate contributed to the design of the study and to the performance of the experiments, collected the results, contributed to the statistical analysis, interpreted the results, drafted the manuscript, designed the figures and finalised the manuscript with input from all the authors

CHAPTER 1

HORIZON 2020 PROJECT TO FUND THE RESEARCH

PAPER 1

Calvo-lerma, J. et al. (2017) Innovative approach for self-management and social welfare of children with cystic fi brosis in Europe : development, validation and implementation of an mHealth tool (MyCyFAPP). Br. Med. J. Open 7:e014931

PAPER 1

INNOVATIVE APPROACH FOR SELF-MANAGEMENT AND SOCIAL WELFARE OF CHILDREN WITH CYSTIC FIBROSIS IN EUROPE: DEVELOPMENT, VALIDATION AND IMPLEMENTATION OF AN MHEALTH TOOL (MyCyFAPP)

Joaquim Calvo-Lerma[a,b*], Celia P. Martinez-Jimenez[c,d], Juan-Pablo Lázaro-Ramos[e], Ana Andrés[b], Paula Crespo-Escobar[a], Erlend Stav[f], Cornelia Schauber[g], Lucia Pannese[h], Jessie M. Hulst[i], Lucrecia Suárez[j], Carla Colombo[k], Celeste Barreto[l], Kris de Boeck[m] and Carmen Ribes-Koninckx[a*]

on behalf of MyCyFAPP.

[a] Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell 106. 46026 Valencia (Spain).

[b] Instituto de Ingeniería de Alimentos para el Desarrollo. Universitat Politècnica de València. Camino de vera s/n. 46022 Valencia (Spain)

[c] University of Cambridge, Cancer Research UK Cambridge Institute, Robinson Way, Cambridge, CB2 0RE (UK)

[d] Welcome Trust Sanger Institute, Welcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, (UK)

[e] Soluciones Tecnológicas para la Salud y el Bienestar. Ronda Auguste Luis Lumiere 23 nave 13, Parque Tecnologico, 46980 Paterna (Spain).

[f] STIFTELSEN SINTEF. P.O. Box 4760 Sluppen, NO-7465 Trondheim (Norway)

[g] YOUSE GmbH. Kyreinstraße 18, 81371 München (Germany)

[h] Imaginary SRL. Piazza Caiazzo, 3 20124 Milano (Italy).

[i] Erasmus Medical Center, Sophia Children's Hospital. Postbus 2040 3000 CA Rotterdam Rotterdam (The Netherlands).

[j] Servicio Madrileño de Salud - Hospital Universitario Ramón y Cajal. Km. 9,100,, Ctra. Colmenar Viejo, 28034 (Spain).

[k] Università degli Studi di Milano. Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico. Via Commenda 9,Via Festa del Perdono, 7, 20122 Milan (Italy).

[l] Associação Portuguesa para a Investigação e Desenvolvimento da Faculdade de Medicina. Av. Prof. Egas Moniz 1600-190 Lisbon (Portugal).

[m] Department of paediatrics, University hospital of Leuven, University of Leuven. Herestraat 49, 3000 Leuven (Belgium)

ABSTRACT

Introduction: For the optimal management of children with Cystic Fibrosis there are currently no efficient tools for the precise adjustment of pancreatic enzyme replacement therapy, neither for advice on appropriate dietary intake, nor for achieving an optimal nutrition status. Therefore, we aim to develop a mobile application that ensures a successful nutritional therapy in children with Cystic Fibrosis.

Methods and analysis: A multidisciplinary team of twelve partners coordinate their efforts in nine work-packages that cover the entire so called "from lab to market" approach by means of an original and innovative co-design process. A cohort of 200 patients with Cystic Fibrosis aged 1-17 years old are enrolled. We will develop an innovative, clinically tested mobile Health application for patients and health professionals involved in cystic fibrosis management. The mobile application integrates the research knowledge and innovative tools for maximising self-management with the aim of leading to a better nutritional status, quality of life and disease prognosis. Bringing together different and complementary areas of knowledge is fundamental for tackling complex challenges in diseases' treatment, such as optimal nutrition and pancreatic enzyme replacement therapy in Cystic Fibrosis. Patients are expected to benefit the most from the outcomes of this innovative project.

Ethics and dissemination: The project is approved by the Ethics' Committee of the coordinating organisation, Hospital Universitari La Fe (Ref: 2014/0484). Scientific findings will be disseminated via journals and conferences addressed to clinicians, food scientists, Information and Communications Technology experts and patients. The specific dissemination working group within the Project will address the wide audience communication through the website (www.mycyfapp.eu), the social networks and the newsletter.

Keywords: Cystic Fibrosis, paediatrics, APP, mHealth, PERT, nutrition, self-management

Strengths and limitations of this study

- Innovative evidence-based method for Pancreatic Enzyme Replacement Therapy adjustment and self-management by means of a mobile application.
- Multidisciplinary team of experts for an integrative and co-designed patients-directed approach.
- Envisaged medium to long-term market uptake of the resulting mobile health application.
- Limited but statistically significant number of patients from 5 European countries will be included in the clinical validation.

1. INTRODUCTION

Cystic Fibrosis (CF) is the most common life-threating autosomal inherited disease in Europe, with over 38.000 cases of CF currently registered in Europe [1]. Along with pulmonary dysfunction and recurrent lung infections, the majority of patients (85%) suffer from lifelong pancreatic insufficiency (PI), which leads to maldigestion of foods and malabsorption of nutrients, especially lipids. In fact, pancreatic enzyme deficiency is occurring in approximately 50 % of infants by the age of two with a further 28% of the cases developing pancreatic insufficiency (PI) in early childhood [2]. These malfunctions secondarily cause malnutrition, fat-soluble vitamin deficiencies, and gastrointestinal complaints.

There is high-grade evidence that maintaining normal growth and nutrition adds 10 years more to the median survival since close relationship between pulmonary function and nutritional status has been repeatedly ascertained [2] [3] [4].

Malnutrition and growth stunting can only be avoided by accurate Pancreatic Enzyme Replacement Therapy (PERT) and close nutritional follow up, as well as, by early nutritional support and intervention. Nowadays, PERT consists of oral supplements containing a mixture of pancreatic enzymes - amylases, proteases and especially lipases - that have to be taken with every meal, while nutritional therapy relies on a high-

energy and high-fat diet [5] [6] [7] [8] [9]. However, at present there is a lack of evidence-based methods to adjust PERT dosing and there are few handy tools or resources adequately available to promote a balanced and adapted diet (**Figure 4.1**) [10] [11] [12].

Current recommendations for PERT-dose adjustment rely on low level of evidence [13] and counsel a number of Units of Lipase per gram of lipids. This means that in every meal, fat content should be known by the patient to estimate the corresponding PERT dose. The only way to achieve this would be by roughly estimating fat content from nutritional information databases and those should be easily available for patients. This approach is challenging for the patients and imprecise to maintain satisfactory levels of fat absorption. In this regard, clinical trials aimed at elucidating maldigestion in CF have led to inconsistent conclusions [11]. Therefore, the demand of an evidence-based criterion for PERT adjustment has been highlighted [10] [11] [14], and the corresponding development of new innovative tools is imperative.

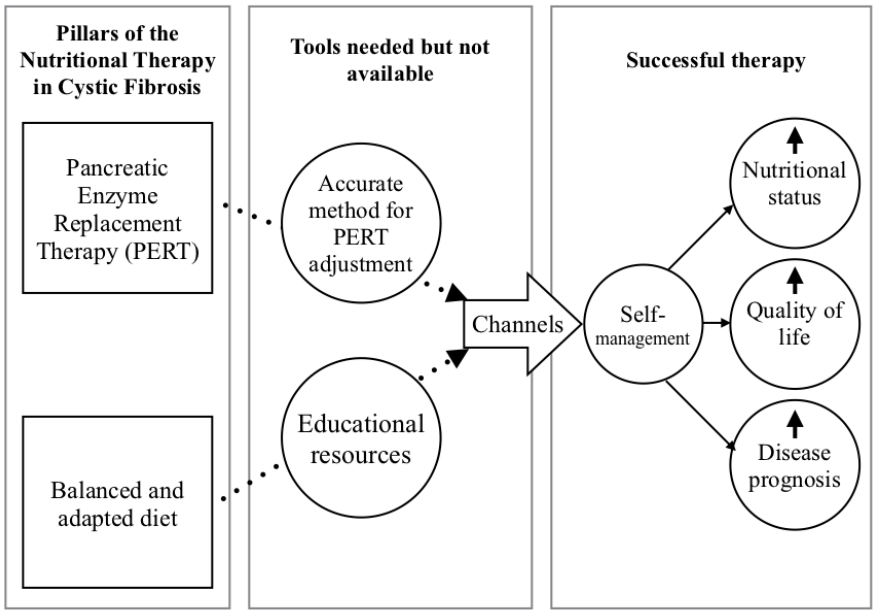


Figure 4.1. Overview of current nutritional therapies in Cystic Fibrosis and the tools needed for successfully achieving a good nutritional status, quality of life and disease prognosis

Dietary lipids need to be accessible to digestive enzymes so that digestion and absorption can occur. The food matrix is dissociated through the digestion process thus allowing the release of the embedded lipids and the access of the enzymes (lipases) to their substrates (lipids) [5] [15]. Recent advances in food science research revealed that the different food structures modulate fatty acids release during digestion and their final metabolic fate [16] [17] [18]. In addition, pancreatic lipase exhibits different hydrolytic activity depending on intra-molecular structure of the lipids [15] [19] [20]. Therefore, lipolysis may cause different kinetics of release of absorbable fatty acids. This can be translated into different enzymatic dosage depending on the inherent-to-food characteristics, so nutrition and dietary habits play a key role in PERT effectiveness [21] [22].

Moreover, the lack of appropriate tools and resources for the nutritional management can impair quality of life and lead to a lack of treatment adherence. For instance, if an incorrect nutritional behaviour or an inadequate PERT dosage occurs, the most likely scenario is that it will occur repeatedly and, in the majority of the cases it will not be detected and corrected until the next contact at the CF Unit. This could lead to long periods of omissions and/or wrong decisions. Consequently, the small daily actions related to nutrition that contribute to the overall disease prognosis would not be optimally used to improve the health status.

Hence, nutritional treatment in CF can be considered as one of the ideal targets of mobile health (mHealth) and patients' self-management. In fact, CF is one of the most representative examples in which patients' monitoring and self-management can lead to a great improvement in the evolution and prognosis of the disease. Among other priorities in health, the current European Union's Research and Innovation Programme, Horizon 2020, strongly supports that current and future lines of research and technological development should be focused on this area [www.ec.europa.eu]. In this framework, MyCyFAPP Project (www.mycyfapp.eu) has been granted to develop an innovative approach focused on paediatric children with CF, self-management of

nutrition and PERT by means of a mobile application (APP) linked to a web-based professional management tool.

The objective of the present work is to describe the overall approach and study design of MyCyFAPP Project as an example of multidisciplinary research and innovation project in mHealth.

2. METHODS

2.1. The Consortium

The Consortium was established in 2015 with the signature of the Grant Agreement with the European Commission. The multidisciplinary research team is comprised of nutritionists-dieticians, paediatric gastroenterologists and pulmonologists, food engineers, IT experts, game developers, software developers, psychologists, sociologists, biologists and patients' representatives. We have brought together our expertise to ensure the successful development of the project through a holistic and integrative approach of the different and complementary areas of knowledge and experts included.

There are twelve organisations involved: six clinical partners linked to their corresponding Research Institutes or Foundations, three small-medium enterprises (SMEs) related to mHealth, one ICT Research Institute, one food technology Research Institute and the European Federation of Patients with CF (**Table 4.1**).

Table 4.1. List of Participating Organisations in MyCyFAPP Project

Country	Organisation	Type of activities
Spain	Instituto de Investigación Sanitaria La Fe	Non-profit organisation pursuing the fostering and promoting of excellent research, scientific and technological knowledge and the translation to the productive sector. It manages research activities of Hospital La Fe, where the regional CF Unit is the reference.

Country	Organisation	Type of activities
Spain	Soluciones Tecnológicas para la Salud y el Bienestar (TSB)	R&D and innovation SME focused on knowledge-intensive solutions for health care and wellbeing.
Germany	YOUSE GmbH	Interdisciplinary SME working on increasing the usability and user experience of products and services.
Italy	Imaginary SRL	Experienced SME in creativity and innovation backed by solid technical competence and an understanding of the commercial potential of serious games and gamification.
Norway	STIFTELSEN SINTEF	Research organisation with expertise within user-centred design, software architecture, software development methods, mobile and social computing and evaluation of technology
Spain	Universitat Politècnica de València – Instituto de Ingeniería de Alimentos para el Desarrollo	University Research Institute focused on Food Engineering. It applies its strong experience in industrial food processing to the area of the digestive food processing, involved in numerous collaborative projects between the industry and academia.
Belgium	University of Leuven	The CF reference center is based at the University Hospital of Leuven and has a strong research focus since many years.
Portugal	Associação Portuguesa para a Investigação e Desenvolvimento da Faculdade de Medicina	It is the funding body that supports medical research in the Hospital de Santa Maria. The CF team conforms the reference unit in the country.

Country	Organisation	Type of activities
Italy	Università degli studi di Milano	Research group linked to the Ospedale Maggiore Policlinico with a wide experience in CF multicentre projects, which is the largest CF reference unit.
The Netherlands	Erasmus Medical Center, Sophia Children´s Hospital Rotterdam	The hospital embraces the reference CF unit for children in the region. Medical team has a commitment with science and research integrity.
Spain	Servicio Madrileño de Salud. Hospital Universitario Ramón y Cajal	The hospital is one of the reference CF unit for children in the region. Medical team has a broad experience in clinical trials and research in the field of CF
Belgium	Cystic Fibrosis Europe	It is the representation of the Patients Organisations in Europe, which is actively involved in dissemination of CF activities and has been playing a key role in EU research projects.

2.2. Funding

MyCyFAPP Project is funded by the European Union through Horizon 2020 Research and Innovation Programme (PHC-26-2014: Self-management of health and disease: citizen engagement and mHealth) under grant agreement No 643806.

2.3. Study design

The 4-year-long project (1st of January 2015 to 31st of December 2018) is constructed on 9 interrelated work packages (WP) (**Figure 4.2**). Four multidisciplinary work-packages (1, 2, 3, 4) set the ground and generate the necessary knowledge and resources to develop the APP. A central technical WP (5) integrates the information in the development of the different software tools. These tools are thereafter tested for

impact through a European Multicentre clinical trial (WP 6) and once the ICT tool is validated another WP (8) takes care of bringing the tool to the market by following different business models. Along the whole Project a specific WP (7) ensures the dissemination of the project to the very wide spectrum of audiences and another one is devoted to the coordination of the Consortium and the management of the implementation.

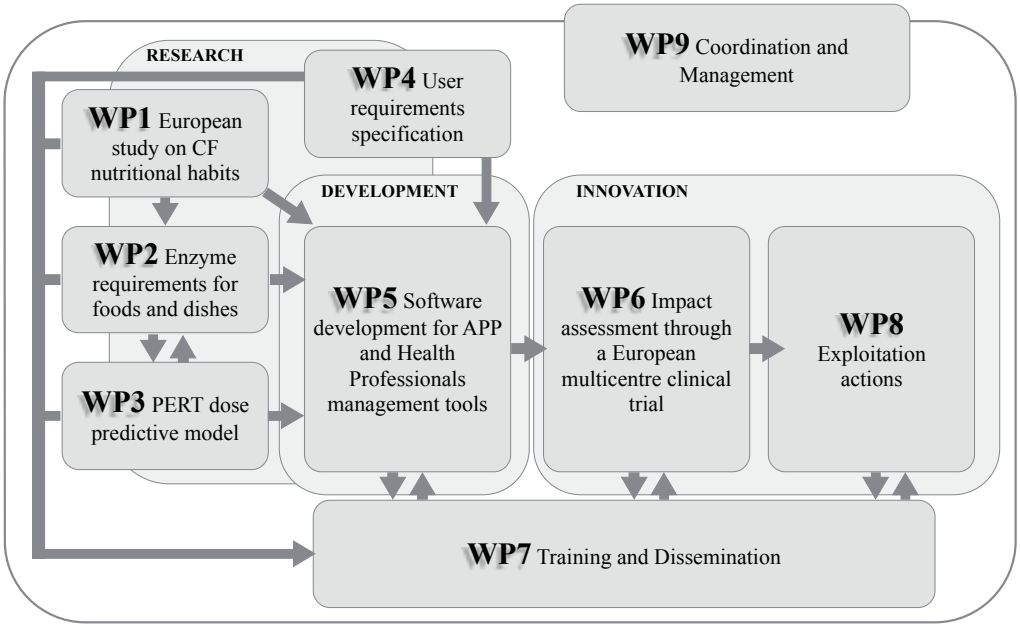


Figure 4.2. General overview and interrelation of work packages (WP)

2.4. Work Packages underpinning the Project

2.4.1. European Study on Dietary Habits in children with Cystic fibrosis (WP1)

One of the first actions of the project aims at obtaining information related to nutritional habits and dietary assessment of CF children in the participating countries. It is used to establish the current nutritional habits of CF children, PERT dosage, nutritional status and dietary assessment as a ground setting. Final milestone is then

the generation of educational tools and resources for a customised nutritional self-management of the disease and patients' empowerment.

2.4.2. In vitro assessment of enzyme requirements for foods and dishes (WP2)

In parallel to the development of the European Survey, we have set up a methodology to *in vitro* simulate digestion of a wide range of foods and meals under standardised CF gastrointestinal conditions. It allows for characterising inherent-to-food factors (chemical composition, molecular structure of lipids, food matrix) and gastrointestinal conditions (composition of digestive fluids and pH of the digestive environment), which affect fatty acids release and enzyme activity. The ultimate goal is to apply these results for determining the optimal PERT doses for foods and meals. They conform a key database supporting the mathematical algorithm.

2.4.3. Development of the PERT dose predictive model (WP3)

We conduct a pilot study with the enrolled children with CF. They follow a fixed menu consisting of a selection of foods and fixed enzyme doses according to the *in vitro* studies (theoretical optimal dose, TOD). Analyses of fat in stools reveal the degree of effectiveness of the predicted dose in each individual.

Biostatistical modelling of the results determines an individual correction factor (ICF) calculation that will be able to correct the *in vitro* dose, for any other meal (even not tested in the pilot study). Thus, from WP2 the TOD estimates the requirements of PERT considering food characteristics. Then, from WP3, the ICF will adjust the TOD according to patients' individual characteristics. These two key elements conform the predictive model, which calculates for each patient an Individual Optimal Dose (IOD).

2.4.4. User requirements specification for Cystic Fibrosis self-management (WP4)

User requirements describe how software solutions work in a certain context of use; how the end users will benefit from it; how the application is managed and maintained; and how it is technically and organizationally deployed. As already mentioned,

MyCyFAPP is not only an ecosystem of APPs, but also a number of tools and components devoted to support the execution of those APPs.

It is critical to gather a multidisciplinary team (developers, clinical partners, psychologists, experts in user experience and acceptance, paediatric and adult end users and patients' associations) to define in detail what the mobile applications will do, and how the clinical processes implemented through the web professional tool will be perceived by the users, both children and care givers. With the goal to maximize the opportunities for further adoption, MyCyFAPP has selected a methodology for the identification of user requirements called "co-creation".

A series of activities including interviews, focus groups and hands-on workshops to establish the needs and preferences regarding the APP usage will be conducted. We establish 5 focus groups (3 patients and 2 parents): patients aged >16 years, patients aged 12-16 years, patients <12 years, parents of patients aged 12-16 years and parents of patients younger than 12 years. The APP will have different functions according to the role and responsibility of the target group in the self-management.

The results will be translated into tailored interfaces and will be easily accessible and user-friendly for the different target populations.

2.4.5. Software development of APP and health professional management tool (WP5)

The results from WP4 are translated into technical specifications, and finally to software mobile and web applications. To this purpose the system architecture, technical specifications, integration plan and software testing strategy is defined. Finally, after software development for full CF self-management, the implementation and integration of the algorithm developed in WP3 and the other resources developed in WP1 are conducted. At that point, the overall system will be delivered for the clinical trial in WP6.

2.4.6. Impact assessment through a European Multicentre Clinical Trial (WP6)

We will carry out a European multicentre clinical trial to assess the impact derived from the utilisation of the APP on children's quality of life (especially related to nutrition and gastrointestinal complaints), nutritional status and healthcare utilisation. A cohort of 200 patients will be recruited. The sample size was estimated using Monte Carlo simulations assuming normally distributed variables, and aiming for a precision of ± 10% for each variable. A validation step is crucial for implementing MyCyFAPP in the usual clinical practice and transferring the self-management utility to patients with CF.

2.4.7. Training and Dissemination (WP7)

This WP embraces a double scope. Training activities are aimed at achieving patient's engagement in self-management of their own disease so specific workshops and webinars are scheduled prior to the start of the clinical trial addressing both patients and health professionals.

Dissemination pursues the Project's awareness, through all media channels, among the key stakeholders: patients and their families, patients' associations, health authorities, professionals from the different disciplines involved in the project, the industry and the general public. Overall it targets the successful implementation of MyCyFAPP.

2.4.8. Exploitation actions (WP8)

This WP takes care of the exploitation of the final product and the Intellectual Property Rights (IPR) protection plans envisaged in the project. Specific actions include the identification of business models for the exploitation of project's outcomes, the definition and execution of the strategy for exploitation and the coordination of the exploitation activities with disseminations to maximise the impact and awareness of the project.

2.4.9. Coordination and management (WP9)

It is aimed at orchestrating all the activities and partners of the project towards the successful implementation of the action and the reach of the goals and milestones.

3. EXPECTED RESULTS

MyCyFAPP project pursues a final scenario where children with CF and their families and, the health professionals can jointly and barriers-free manage the treatment of the disease. On one side, patients and families count on the APP to self-manage nutrition and PERT and, on the other side, health professionals use the professional tool to supervise and monitor patients' progress, ensuring feedback between the two parts when needed. This process is possible thanks to the specifically developed procedures and tools (features) that are addressed in the framework of the project from a rigorous scientific approach, responding to the current gaps on the resources needed but not available for a successful nutritional therapy (**Figure 4.3**).

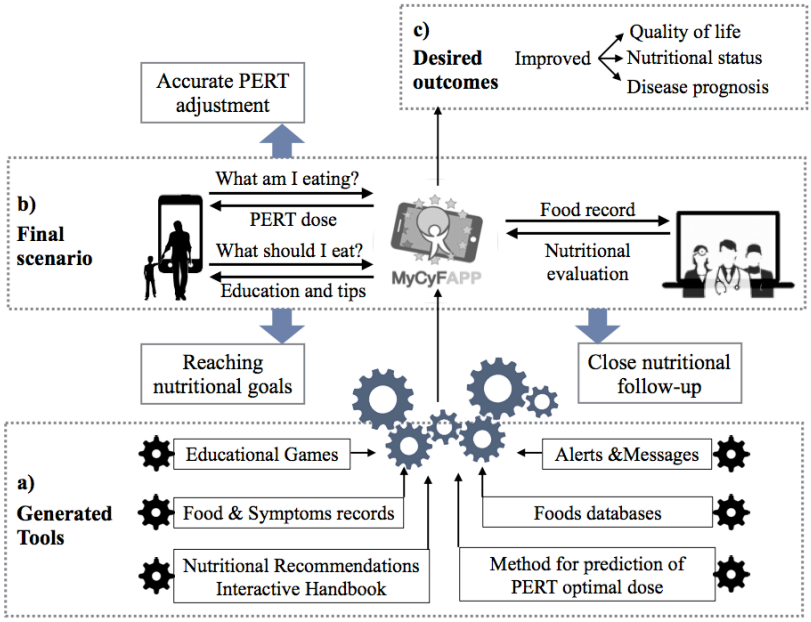


Figure 4.3. Summary of the Project: generated tools (a), expected final scenario at the end of the Project (b) and desired outcomes (c)

3.1. Tools and resources for MyCyFAPP

Throughout the first WPs of the project, we conduct research that results in the generation of the needed tools and resources for the APP (**Figure 4.3a**). The "mathematical predictive model" of the optimal dose of enzymes is the main feature, tackling the currently existing gap to successfully adjust PERT. It is fed by the "theoretical PERT doses database" including the optimal dose to digest a particular food or meal plus the individual correction factor of each patient. It becomes functional when the users indicate the foods consumed and the amounts. A full and "interactive nutritional recommendations handbook" is also available in the APP supporting children's dietary habits towards avoiding and correcting nutritional imbalances and reaching the recommendations.

"Food and symptoms record" is automatically generated and stored from the data introduced by the patients into the APP. This feature works thanks to the calculation algorithms and the "foods databases", which include specific foods and meals/recipes according to the survey on nutritional habits and the complete nutritional profile information. The record allows for consulting at any time patients' progress in terms of nutritional composition of their diets, their symptoms and the actions they have performed in the system. "Educational games" are developed in order to convey educational content of the recommendations handbook to the youngest children who cannot consult it. Games also have versions for older patients, these being aimed at consolidating the knowledge learnt by the other features. Finally "alerts and messages" systems smooth the usability of the APP between the two sides of MyCyFAPP – the patients and the clinical teams - making the experience profitable and appealing.

Other specific features will be incorporated in the management system to enable health professionals to play their role: the professional tool. This module contains several features, such as a patients' dashboard displaying a summary of each patient - energy intake, percentage of nutrients, symptoms, number of depositions, etc. - from where patients' profiles (especially focused on nutrition) can be accessed. Then,

“adjustment of parameters” allows for making a more focused follow-up and to set up goals, and the “care plan management module” is to define the overall strategy for patient. Complementarily, an education content management module and a report module are in charge of creating a report to be sent to the patients describing how they fit to their personalized plan. Trough an iterative process with partners and final users, updates and corrections are periodically applied. Thus the final set of features and tools will be decided along the project.

The ultimate goal is to motivate the users to adhere to the plan with positive messages when needed, and proposing new challenges.

3.2. Final scenario

When the APP is ready-to-use (**Figure 4.3b**), patients introduce the food products or dishes and the APP indicates in real-time the optimal PERT dose for the particular meal and considering the individual correction factor of the patient. This at the same time generates in real time a food record and its automatic nutritional report. Complementary patients are already taught and skilled to build up their menus according to the dietary recommendations, and when needed, they are offered to consult suggestions or practical tips.

Some of the functions enabled by the interaction between the patients and the clinical teams include the periodic check of the daily results of the nutritional profile of the diet. The software is programmed to alert patients and medical teams in case of a deviation from general or personalised recommendations (e.g. percentage of lipids does not reach the threshold this week). If a deviation is identified as relevant – according to the definition of a risk and the plan for the patient – the health professionals can be notified, through the professional web tool, and are then responsible to decide which correction procedure has to apply (e.g. consult educational resource number 1.3). For some situations, however, the software is programmed to automatically pop-up reaction messages. Thus, the overall aim is providing feedback and assistance to the patients outside the schedule face-to-face visits.

Of note, the above-described situation is thoroughly assessed through a multicentre clinical trial, that will allow for the identification of errors and the features and procedures showing room for improvement. Therefore, updates and modifications can be applied before upgrading the system to the final and fully functional version. If success in the clinical validation occurs, MyCyFAPP can be able to reach the market by following the defined exploitation plan.

3.3. Desired outcomes

Overall, we expect that the mHealth solution contributes to reach project's goals: an evidence-based method for PERT adjustment, reaching nutritional goals and close nutritional follow-up. The desired outcomes derived from its long-term utilisation are a triple improvement: quality of life specifically related to gastrointestinal symptoms, nutritional status and disease prognosis (**Figure 4.3c**).

4. CONCLUSION

Through MyCyFAPP we have brought together highly experienced professionals from various European countries with different areas of knowledge to jointly address the challenges faced by adequate nutrition and PERT in the management of CF. We mainly tackle two gaps within the project: first, we develop from scratch the required tools for effective PERT and nutritional therapy; secondly, we make the tools available to patients enabling effective adherence to the disease treatment through self-management but still, when needed maintaining a close and dynamic interaction with the medical teams throughout the mobile health tool.

The beneficiaries of the projects' results comprise patients, caregivers, families and healthcare professionals. MyCyFAPP is designed in a tailored way and clinically tested for CF self-management and monitoring. Additionally, MyCyFAPP has a pivotal role as a decision support system and provides a solution to the current gaps in the treatment. The participating SMEs and business models will ensure the commercial exploitation of the results, the market uptake and the MyCyFAPP distribution for the

benefit of the patients. We envisage a prominent impact on nutritional status, quality of life and overall disease prognosis in the near future.

"When people ask me to provide an example of how patients, caregivers, researchers, a Foundation, NIH and industry can all work together to find cures, I point to cystic fibrosis. It's the very best example."

FRANCIS S. COLLINS, M.D., Ph.D. Director of the National Institutes of Health and a member of the international team that discovered the CF gene.

ACKNOWLEDGEMENTS

Authors of this paper, on behalf of MyCyFAPP consortium, acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme for funding the project (ref. 643806).

Contributorship statement

J Calvo-Lerma, CP Martínez-Jimenez, A Andrés, JP Lázaro-Ramos and C Ribes-Konickx designed the research. E Stav, P Crespo-Escobar, C Schauber, L Pannese, JM Hulst, L Suárez, C Colombo, C Barreto and K de Boeck contributed to the review and improvement of the project design. J Calvo-Lerma, CP Martínez-Jimenez, A Andrés, JP Lázaro-Ramos and C Ribes-Konickx drafted the first version of the manuscript and revised it critically for important intellectual content, and E Stav, P Crespo-Escobar, C Schauber, L Pannese, JM Hulst, L Suárez, C Colombo, C Barreto and K de Boeck contributed to the revision of the manuscript ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All the authors approved the final version of the work.

Competing interests

All authors declare that there are no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

Funding

Horizon 2020 Research and Innovation Programme of the European Union. PHC-26-2014 call Self management of health and disease: citizen engagement and mHealth, MyCyFAPP 643806.

Data sharing statement

The project is currently in a pre-results stage.

6. REFERENCES

1. Colombo, C., & Littlewood, J. (2011). The implementation of standards of care in Europe: state of the art. *Journal of Cystic Fibrosis*, *10*, S7-S15.
2. Gaskin, K. J. (2013). Nutritional care in children with cystic fibrosis: are our patients becoming better&quest. *European journal of clinical nutrition*, *67*(5), 558-564.
3. Stephenson, A. L., Mannik, L. A., Walsh, S., Brotherwood, M., Robert, R., Darling, P. B., ... & Stanojevic, S. (2013). Longitudinal trends in nutritional status and the relation between lung function and BMI in cystic fibrosis: a population-based cohort study. *The American journal of clinical nutrition*, *97*(4), 872-877.
4. Kerem, E., Viviani, L., Zolin, A., MacNeill, S., Hatziagorou, E., Ellemunter, H., ... & Olesen, H. (2014). Factors associated with FEV1 decline in cystic fibrosis: analysis of the ECFS Patient Registry. *European Respiratory Journal*, *43*(1), 125-133.

5. Aaron Fieker, Jessica Philpott, Martine Armand. Enzyme replacement therapy for pancreatic insufficiency: present and future.. Clinical and Experimental Gastroenterology, Dove Medical Press, 2011, 4 (4), pp.55-73
6. Sikkens, E. C., Cahen, D. L., Kuipers, E. J., & Bruno, M. J. (2010). Pancreatic enzyme replacement therapy in chronic pancreatitis. Best Practice & Research Clinical Gastroenterology, 24(3), 337-347.Whitcomb 2010
7. Wilschanski, M., & Durie, P. R. (2007). Patterns of GI disease in adulthood associated with mutations in the CFTR gene. *Gut*, *56*(8), 1153-1163.
8. Woestenenk, J. W., van der Ent, C. K., & Houwen, R. H. (2015). Pancreatic enzyme replacement therapy and coefficient of fat absorption in children and adolescents with cystic fibrosis. Journal of pediatric gastroenterology and nutrition, 61(3), 355-360.
9. Haupt, M. E., Kwasny, M. J., Schechter, M. S., & McColley, S. A. (2014). Pancreatic enzyme replacement therapy dosing and nutritional outcomes in children with cystic fibrosis. The Journal of pediatrics, 164(5), 1110-1115.
10. Borowitz, D., Durie, P. R., Clarke, L. L., Werlin, S. L., Taylor, C. J., Semler, J., ... & Baker, R. D. (2005). Gastrointestinal outcomes and confounders in cystic fibrosis. Journal of pediatric gastroenterology and nutrition, 41(3), 273-285.
11. Fieker, A., Philpott, J., & Armand, M. (2011). Enzyme replacement therapy for pancreatic insufficiency: present and future. Clinical and experimental gastroenterology, 4(4), 55-73.Ex 9 turk et al
12. Borowitz, D., Gelfond, D., Maguiness, K., Heubi, J. E., & Ramsey, B. (2013). Maximal daily dose of pancreatic enzyme replacement therapy in infants with cystic fibrosis: a reconsideration. *Journal of Cystic Fibrosis*, *12*(6), 784-785.
13. Turck, D., Braegger, C. P., Colombo, C., Declercq, D., Morton, A., Pancheva, R., ... & Schneider, S. M. (2016). ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. Clinical Nutrition, 35(3), 557-577.

14. Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: proposal for a standardised pH-stat method. Food Chemistry, 126(2), 498-505.
15. Hunter, J. E. (2001). Studies on effects of dietary fatty acids as related to their position on triglycerides. Lipids, 36(7), 655-668.
16. Stuknytė, M., Cattaneo, S., Masotti, F., & De Noni, I. (2015). Occurrence and fate of ACE-inhibitor peptides in cheeses and in their digestates following *in vitro* static gastrointestinal digestion. Food chemistry, 168, 27-33.
17. Parada, J., & Aguilera, J. M. (2007). Food microstructure affects the bioavailability of several nutrients. Journal of food science, 72(2), R21-R32.Asfd
18. Singh, H., Ye, A., & Horne, D. (2009). Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. Progress in Lipid Research, 48(2), 92-100.Asdf
19. Zhu, X., Ye, A., Verrier, T., & Singh, H. (2013). Free fatty acid profiles of emulsified lipids during in vitro digestion with pancreatic lipase. Food chemistry, 139(1), 398-404.
20. Lesmes, U., & McClements, D. J. (2012). Controlling lipid digestibility: Response of lipid droplets coated by β-lactoglobulin-dextran Maillard conjugates to simulated gastrointestinal conditions. Food Hydrocolloids, 26(1), 221-230.Asdf
21. Giang, T. M., Gaucel, S., Brestaz, P., Anton, M., Meynier, A., Trelea, I. C., & Le Feunteun, S. (2016). Dynamic modeling of in vitro lipid digestion: Individual fatty acid release and bioaccessibility kinetics. *Food chemistry*, *194*, 1180-1188.
22. Nieva-Echevarría, B., Goicoechea, E., Manzanos, M.J. (2016). A study by 1H NMR on the influence of some factors affecting lipid in vitro digestion. *Food Chemistry, 211,* 17-26.

CHAPTER 2

CURRENT SITUATION OF NUTRITION AND PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS CHILDREN IN EUROPE

PAPER 2

Calvo-Lerma, J. et al. (2017). Pancreatic enzyme replacement therapy in cystic fibrosis: dose, variability and coefficient of fat absorption. Revista española de enfermedades digestivas, 109(10), 684-689.

PAPER 3

Calvo-Lerma, J. et al. (2017). Nutritional status, nutrient intake and use of enzyme supplements in paediatric patients with Cystic Fibrosis; a European multicentre study with reference to current guidelines. Journal of Cystic Fibrosis, 16(4), 510-518.

PAPER 4

Calvo-Lerma, J. et al. (2018). Children with Cystic Fibrosis present with dietary imbalances: a European multicentre comparison of food groups and origin of nutrient intake. Journal of The Academy of Nutrition and Dietetics (under review).

PAPER 2

PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS: DOSE, VARIABILITY AND COEFFICIENT OF FAT ABSORPTION

Joaquim Calvo-Lerma[a,b*], Sandra Martinez-Barona[a*], Etna Masip[a,c], Victoria Fornés[a] and Carmen Ribes-Koninckx[a,c]

[a] Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell 106, Torre A planta 0. 46026 Valencia (Spain)

[b] Instituto de Ingeniería de Alimentos para el Desarrollo. Universitat Politècnica de València. 46022 Valencia (Spain)

[c] Hospital Universitari i Politècnic La Fe. Avda. Fernando Abril Martorell 106, Torre C planta 2. 46026 Valencia (Spain)

* Corresponding author: Joaquim Calvo-Lerma (joaquin_calvo@iislafe.es)

ABSTRACT

Objectives: pancreatic enzyme replacement therapy (PERT) remains a backbone in the nutritional treatment of Cystic Fibrosis. Currently there is a lack of an evidence-based tool that would allow dose adjustment. To date, no studies have found an association between PERT dose and fat absorption. Therefore, the aim of the study was to assess the influence of both the PERT dose and the variability in this dose on the coefficient of fat absorption (CFA).

Methods: retrospective longitudinal study including 16 paediatric patients (192 food records) with three consecutive visits to the hospital over a twelve-month period. Dietary fat intake and PERT were assessed through a 4-day food record and fat in stools was determined by means of a 3-day stools collection. A beta regression model was built up to explain the association between the CFA and the interaction between the PERT dose (lipase units/ g dietary fat) and the variability in the PERT dose (standard deviation).

Results: the coefficient of fat absorption increased with the PERT dose as long as the variability in the dose was low. In contrast, even at the highest PERT dose values, the CFA decreased when the variability was high. The confidence interval showed a suggesting association, although the analysis was not statistically significant.

Conclusion: the variability in the PERT dose adjustment should be taken into consideration when performing studies on PERT efficiency. A clinical goal should be trying to maintain a constant PERT dose rather than only trying to find an optimal figure.

Keywords: Cystic fibrosis; enzyme to substrate ratio; pancreatic enzyme replacement therapy (PERT); enzymatic supplements; coefficient of fat absorption; variability.

Abbreviations: Cystic Fibrosis (CF), Pancreatic Insufficiency (PI), Pancreatic Enzyme Replacement Therapy (PERT), Coefficient of Fat Absorption (CFA), Faecal Elastase (FE1), Body Mass Index (BMI), Forced Expiratory Volume (FEV1%), Grams (g), Lipase Units (LU), Enzyme to Substrate ratio (E/S), Interquartile Range (IQR), Standard Deviation (SD).

1. INTRODUCTION

Cystic Fibrosis (CF) is the most common life threatening genetic disease in Europe (1). Although symptoms may differ widely from one patient to another, there are two systems, which are almost unequivocally affected, these being the pulmonary and gastrointestinal tract (2).

Eventually, up to 90-95% of patients with CF become pancreatic insufficient (PI) (3-5) leading to malabsorption and secondarily to malnutrition of carbohydrates, protein and especially fat (6,7), compromising the nutritional status of patients (3). Reversing this situation is beneficial above all because an optimal nutritional status is

concurrently associated to better pulmonary outcomes. As respiratory complications are the main cause of morbi-mortality in CF patients (1), maintaining an adequate nutritional status improves the overall prognosis and survival (7-9).

Nutritional intervention in CF patients is based on dietary recommendations of energy intake and nutrients distribution (10), fat-soluble vitamin supplementation and life-long exogenous pancreatic replacement therapy (PERT), which are generally required by almost every CF patient (8). PERT has been proven an effective therapy for improving nutrient absorption, especially that concerning lipid (6,8) although in some cases malabsorption still persists even though PERT is instituted (3).

The evaluation of fat malabsorption is in practice obtained by means of a seventy-two-hour stool collection which estimates fat output and dietary records which enable the calculation of fat intake, allowing altogether the assessment of the coefficient of fat absorption (CFA) (4). The estimated mean value of the CFA in healthy subjects is of 90-95% (8) whilst an adequate PERT dosage in CF patients will result in a fat absorption of more than 85% (4,7).

However, although theoretical PERT adjustment recommendations are extensively disseminated, these have limited scientific foundation (10). Currently, the only two criteria for adjusting PERT are the fat content of the meal and the body weight. Although recent studies have proven there is no clear co-relationship between PERT dosage and CFA, other variables have not been considered. Therefore, the aim of the present study was to assess the long-term influence of both the dose of PERT and the variability in this dosage on the coefficient of fat absorption.

2. METHODS

2.1. Subjects and study design

We conducted a retrospective, observational study including 16 Spanish children with CF, followed-up in the paediatric CF Unit of Hospital Universitario y Politécnico La Fe (Valencia, Spain).

All children included had a confirmed CF diagnosis by means of a positive sweat chloride test and the presence of 2 known CF mutations. None of the patients underwent newborn screening, as it had not yet been instituted at the time they were born. In order to be included in the study being pancreatic insufficient (faecal elastase values (FE1) <200mcg/g of stool), receiving PERT and being aged 1 to 17 was required. Organ transplantation was the only exclusion criterion contemplated. Patients attended three visits, each of them separated by a period of six months. In each appointment demographic and clinical data were collected.

2.2. Clinical measurements

Demographic variables included age, gender, and genotype (severe genotypes were considered, those presenting homozygous mutations in F508-del and G542X mutations). Anthropometric parameters included body weight (expressed in kg), height (expressed in cm) and body mass index (BMI) (expressed as body mass (weight) divided by the square of the body height, expressed in units of kg/m^2). Z-scores (standard deviation) for weight, height and BMI, were calculated, based on the Center for Disease Control (CDC) growth charts. Forced expiratory volume (FEV1) values were selected as representative measures of pulmonary function and were expressed as percentages.

Every patient filled in a total of 3 dietary records each one of them consisting on 4 days (3 of them weekdays and 1 of them a weekend day) spaced each one of them by a 6-month period. Therefore, dietary records consisted on a 4-day food dairy template filled in by the patient/ parents in which the following sections were included: 3 main meals (breakfast, lunch, dinner), 3 snacks (morning, afternoon, night) and "others" (extra snacks). For each one of these meals, the following data were introduced: name of meal, ingredients/ products and amount (expressed in grams (g)). During the dietary records, patients were encouraged to continue with their usual dietary habits. PERT dose for every separate meal was also registered in the records.

Dietary records of each patient were transferred to a calculation system which is based on a nutritional composition database made up of >700 items. This system was used to calculate the dietary intake of energy, carbohydrates, fat and protein.

A 72-hour stool collection was obtained for each patient to determine fat excretion. Stool collection was initiated on the second day of the 4-day dietary diary. CFA was calculated with the following formula: fat intake (g) minus fat excreted (g)/fat intake (g), expressed as a percentage.

The study was performed after approval of the Ethics Committee of Medical Research Institute La Fe of the University Hospital La Fe, Valencia (Spain).

2.3. Statistical analyses

The percentage of fat, protein and carbohydrates was obtained for each separate meal and afterwards the mean and median percentage of these macronutrients was calculated for each day and each dietary record.

PERT dose was expressed in terms of Lipase Units per gram of fat and meal (LU/g fat/ meal), also referred as enzyme to substrate ratio (E/S). PERT dose was registered for each meal in the database and the mean and standard deviation (SD) and median and interquartile range (IQR) dose of PERT were calculated per day and per patient (mean value of the 4-day record).

In order to represent the variability in the PERT dose, the variable SD/ES was established, this representing the standard deviation of the mean dose of PERT. Variability was considered as a categorical variable and was defined as medium (represented by the median value), high (represented by the third quartile) or low (represented by the first quartile).

Therefore SD/ES expresses how differently patients have adjusted the dose of PERT with regard to the amount of dietary fat and how this dosage varies from meal to meal and from day to day. For instance, if a patient takes 1000 LU/g of fat at breakfast, 800 LU/g of fat at lunch and 2000 LU/g of fat at dinner, this will suppose a mean E/S of

1266 LU/g and a mean dose variability of ±1616.5 LU/g (this being the standard deviation of the mean dose).

Due to the high variability among our variables and with the intention of approximating our data to a normal distribution for gaining reliability in the model and avoiding bias in the coefficients estimation, logarithms (log) of E/S and SD/ES were used.

In order to search an association between the co-variables percentage of dietary fat, age, gender, severe mutation and the interaction between log (E/S) and log (SD/ES) in CFA a beta regression model was adjusted given the nature of the response variable (percentage). Furthermore, random effect of subject was taken into account because the database is longitudinal (repeated measures). A p-value < 0.05 was considered statistically significant. All the analyses were performed with the statistics R software (version 3.3.1) and glmmADMB package has been used. E/S values = 0 have been excluded.

Coefficients < 1 suggest an inverse or negative relationship between a co-variable and the response variable (CFA) and coefficients >1 indicate a direct or positive effect.

3. RESULTS

3.1. Demographic, clinical and dietary data

A total of 16 patients (9 male) were enrolled in the study. Patients included were born between 1997-2011 and the global mean age was of 8.6 (± 4.1) years. Severe genotypes were present in 8 patients (50% of the sample).

All patients were confirmed to be PI by means of a faecal elastase-1 test (FE1). According to our laboratory reference values, figures <200 μg/g of stool corresponded to PI, figures between 100-200 μg/g of stool corresponded to moderate PI, and figures <100 μg/g of stool represented severe PI. Our laboratory did not detect FE1 values <15 μg/g of stool. All patients included presented severe PI according to the FE1: 10 patients

presented values <15 µg/g of stool and 6 between 15-50 µg/g of stool (mean value of 24.4± 12.28). All of them received PERT.

Patients included maintained an adequate nutritional status with a mean median BMI value of the 3 visits of 15.7kg/m^2 (15.1,17.1). Pulmonary function of all patients, based on FEV1% values was normal, with a mean FEV1% value of 86.77 (±16.88). Mean value of fat in stools was 11.47 (± 7.22) g/24h (**Table 4.2**).

Table 4.2. Clinical and analytical data of patients along the study period

Variable	Visit 1 (M0)	Visit 2 (M6)	Visit 3 (M12)	Global
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
BMI (kg/m^2)	15.96 (15.32, 17.49)	15.48 (14.87, 16.31)	15.45 (15.09, 17.1)	15.79 (15.1, 17.51)
FEV1 (%)	84.77 (±17.32)	91.98 (±18.06)	80.29 (±15.08)	86.77 (±16.88)
Fat in stools (g/24h)	11.22 (±7.27)	11.69 (±7.58)	11.51 (±8.33)	11.47 (±7.72)

IQR interquartile range, *SD* standard deviation

A total of 192 food records were obtained (16 patients, 4-day food record and 3 visits) from which 1152 meals were characterised (5 to 7 meals a day) in terms of enzyme dose, nutritional composition and energy value of the foods registered.

When studying the nutritional composition of the records, we found out that the global mean daily percentage of carbohydrate, protein and fats was 42.35 (±5.67), 18.42 (±3.67) and 39.09 (±4.31) respectively and the global mean energy intake was of 91.7 (±37.23) kcal/kg weight, with minor variations among the three visits.

Considering the total enzyme to substrate ratio (E/S) in every meal, day, visit and patient, we found a median global E/S intake of 719.4 (451.5, 1205) LU/g of fats with an intra-patient variability (SD E/S) of 616.7 (308.1, 1516) LU/g among different meals.

Dietary fat intake and stools analyses allowed for the calculation of 144 CFA values. The global median CFA value of all 3 registrations and for all patients was 89.7% (84.88, 93.31), with very little variability among records (**Table 4.3**).

Table 4.3. Results obtained by means of the 4-day food record

Variable	Visit 1 (M0)	Visit 2 (M6)	Visit 3 (M12)	Global
Energy intake (kcal/kg) *(mean ±SD)*	97.89 (±38.87)	93.53 (±39.69)	84.09 (±34.13)	91.67 (±37.23)
Protein intake (%) *(mean ±SD)*	18.26 (±3.87)	19.01 (±4.05)	18.32 (±3.55)	18.42 (±3.67)
Carbohydrates intake (%) *(mean ±SD)*	40.64 (±3.98)	41.29 (±4.7)	43.19 (±5.27)	42.35 (±5.67)
Fat intake (%) *(mean ±SD)*	40.45 (±2.96)	39.64 (±3.92)	38.06 (±4.35)	39.09 (±4.31)
Enzyme to substrate ratio (E/S) (LU/g fat/meal)	1257.08 (±3555.53)	1037.96 (±1955.42)	1945.4 (±11708.98)	1413.48 (±5739.97)
(mean ±SD)	724.6	647.45	783.7	719.4
(median, IQR)	(390.6, 1265.8)	(410.65, 1015.2)	(526.3, 1351.4)	(451.5, 1205)
Variability in E/S (SD in LU/g fat/meal) *(median, IQR)*	719.44 (340.67, 1178.04)	378.62 (303.65, 811.64)	529.9 (308.05, 3215.2)	616.7 (308.1, 1516)
CFA (%)	86.62	87.6	88.17	87.73
(mean ±SD)	(±9.84)	(±12.64)	(±9.16)	(±10.43)
(median, IQR)	89.1 (84, 92)	91.1 (84.9, 94.2)	89.5 (86.4, 92.4)	89.6 (84.9, 93.3)

3.2. Association of the study variables with the CFA

When applying the linear regression model, a direct relationship was found between the fat intake and the CFA ($p = 0.019$) and also between the presence of severe mutations (including homozygous F508-del and G542X mutations) and the CFA,

although this last association was not found to be significant (p = 0.07). We found no association between age, gender or BMI and the CFA (**Table 4.4**).

Table 4.4. Results of the lineal regression model: effect of the variables assessed on the coefficient of fat absorption (CFA).

Variable	Coefficient	95% Confidence Interval	p-value
Fat intake (g)	1.02	[1.01, 1.04]	**0.019**
BMI	0.96	[0.89, 1.03]	0.304
Gender	1.40	[0.89, 2.18]	0.138
Age	1.03	[0.98, 1.08]	0.183
Severe mutations*	1.45	[0.97, 2.16]	**0.070**
Log(E/S)	1.41	[0.91, 2.17]	0.125
SD log(E/S)	1.49	[1.01, 2.22]	**0.049**
Log(E/S): SD log(E/S)**	0.94	[0.89, 1.01]	**0.101**

CI confidence interval

* F508del homozygous mutations and G520X mutations were considered severe genotypes.

** Interaction of the variables "enzyme to substrate ratio (log E/S)" and "variability in enzyme to substrate ratio (SD log E/S)"

The interaction between the E/S (log E/S) and its variability (SD log E/S) was assessed: the effect on the response variable (CFA) was different when assessing these two variables as an interaction than when assessed separately. The interaction between the PERT dose (log E/S) and its variability (SD log E/S) was assessed: the effect on the response variable (CFA) was different when assessing these two variables as an interaction than when assessed separately. The interaction had a negative effect on CFA (p = 0.101). Thus, even though the effect of the interaction between these two variables was not significant CFA values depend not only on the E/S, but also the dose-effect changes depending on the variability in the dose (**Figure 4.4**). Interpreting the figure some statements can be ruled out: First of all, taking dose into account in those cases when PERT dose was high, the CFA was as optimal as possible when there was a

low variability. Secondly, taking variability into account when E/S remains constant (low variability, i.e. low SD log E/S) the higher the PERT dose (E/S), the higher the CFA. However, if the variability is high (high SD log E/S), even when the PERT dose is high, the CFA is lower than when the variability is low.

Finally, from our results we must highlight that there is an association between the interaction (dosage with dosage variability) and the response variable (CFA). The effect of PERT dose on CFA depends on the individual variability of E/S.

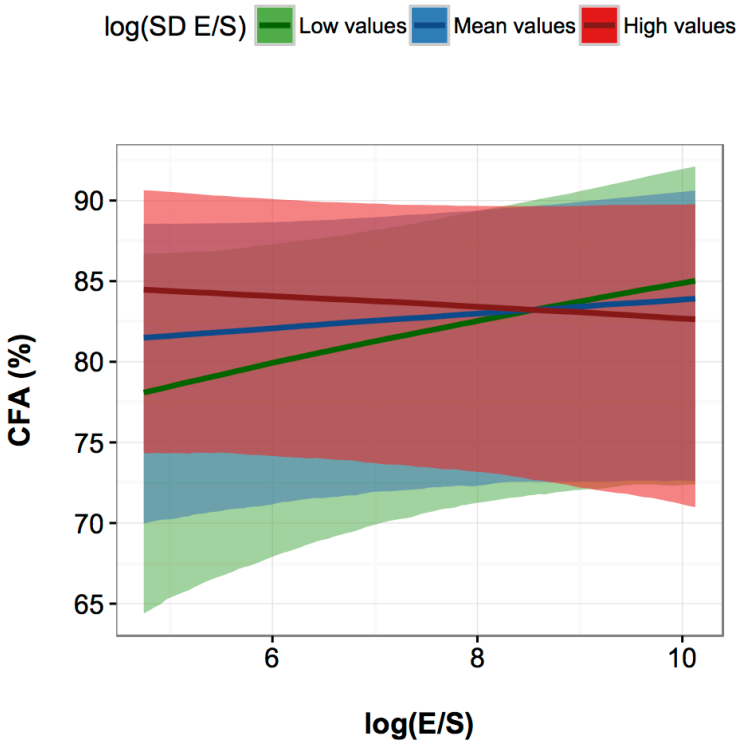


Figure 4.4. Effect of enzyme to substrate ratio (log E/S) on Coefficient of fat absorption (CFA) according to variability on enzyme to substrate ratio intake (log SD E/S)

4. DISCUSSION

In the present study we attempted to identify the effect that PERT dose referred to gram of fat, and its variability, among other factors, have on the CFA in a cohort of 16 pancreatic insufficient paediatric patients with CF.

A genotype-phenotype association has been proven to be positive in cases of pancreatic disease, thus CF patients with severe mutations (types I-III), are more likely to become PI (12,13). In our study we found that those patients presenting mutations involving G542X and homozygous F508-del (mutations belonging to Group I and II respectively) were more likely to present malabsorption and presented a diminished CFA, although these results were not found to be significant.

FE1 is an indirect method, with a high sensitivity for detecting EPI in children (14,15), expressing an excellent co-relation between its values and pancreatic enzyme secretion. In addition, its values seem to be unaffected by PERT (16). However, although all of our patients presented severe EPI according to their FE1 values (all of them <100 μg/g stool) and required exogenous enzymatic supplementation, we saw big differences on their PERT needs, or at least, on their PERT doses (E/S). This suggests that although FE1 enables for a diagnosis and PI classification, it is of limited value for PERT adjustment.

When PI is present, lipid digestion is the most intensively impaired, being in this setting, exogenous supplementation required to compensate for these deficiencies. However, information regarding PERT adjustment is scarce and an identifiable dose-response between enzymes and symptoms has not been demonstrated (3). Dosage recommendations are up to date based on patient's age and body weight (11) and lipase units per gram of dietary fat, nevertheless it has been proven that this is an insufficient evidence-based criterion for a successful adjustment (8). In fact, a recently published study concluded that there is an enormous variability in the response to PERT among patients, with no clear correlation between the CFA and PERT, re-stating assumptions of previous studies in the field (7). Our results demonstrate that the constancy *vs.* the variability in the dose adjustment - in terms of E/S - is to be considered as a variable when performing studies on PERT efficiency.

Moreover, patients usually tend to follow a fixed pattern of PERT dosage for the different meals, regardless of the differences in the macronutrient composition of the food, ignoring the fact that enzyme dosage should match the amount of fat

ingested per meal (4). To reinforce the statement above, several authors (17-19) have declared that in clinical practice some recommend a PERT dose for snacks corresponding to half the dose established for main meals. Other authors suggest that the best control of symptoms is achieved by those patients who auto-adjust PERT according to fat-intake, in contrast to those following fixed-patterns (14). In fact, recent European consensus guidelines recommend a daily enzyme dose which should be adjusted to the amount of fat ingested per meal (2000-4000 Lipase Units (LU) per gram of dietary fat) and increasing upward as needed, with a maximum daily dose of 10000 LU/kg (10). Lipase values exceeding these figures, were formerly related to an increased risk of fibrosing colonopathy, however recent studies question this assertion concluding that the optimal PERT dose for children still remains unidentified (19).

Despite many patients fulfil current recommendations of enzyme dosage (i.e. daily average dosages within the 2000-4000 LU/g fat range) and progress favourably and symptom-free, stool analysis continues revealing, in some cases, malabsorption. This could be explained by the large margin in enzyme recommendations. In addition, these recommended doses are referred to the amount of fat in meals, which may largely vary daily. This raises the suspicion that, besides the enzyme dose, there are other factors that are not being taken into consideration. Among others, the variability of enzyme to substrate ratio (E/S) from one meal to another and from one day to another could be contributing to the result in the coefficient of fat absorption.

In our cohort all patients received an enzyme dose below the current recommendations 2000-4000 LU/g fat, fact that is justified by the adherence to former recommendations (11), which consists of more permissive ranges 500-4000 LU/g fat. Despite this fact, the mean CFA of the cohort was of 87.73% (±10.43), value that is higher than the goal (10).

Finally, our study has some limitations, for instance, we used no dyes for marking stools as referred in other studies (8), fact which reduces the accuracy in the estimation of the CFA. Although we only counted with 16 patients, we obtained a

reasonable number of dietary records and results of fat in stools analyses, enabling for an acceptable statistical analysis.

CONCLUSION

In order to understand the concept of CFA the existing interaction between PERT dosage (in terms of LU/g of fat) and PERT variability should be considered. Although our results were not statistically significant, this could be justified by some methodological limitations and studies with more patients should be considered in the future in order to confirm this association. From our results, we can deduce that the aim in PERT adjustment should not be only to obtain a particular E/S figure, but also to maintain this E/S value constant. We suggest that the step ahead would be to work out the optimal E/S figure for each patient.

REFERENCES

1. O'Sullivan BP, Freedman SD. Cystic Fibrosis. Lancet 2009; 373: 1891-4.
2. Farrel PM, Kosorok MR, Rock MJ et al. Early Diagnosis of Cystic Fibrosis Through Neonatal Screening Prevents Severe Malnutrition and Improves Long-Term Growth. Pediatrics 2001; 107: 1-13.
3. Baker RD. Assessing exocrine pancreatic function: when the best test is not possible. J Gastroenterol Nutr Pediatr 2013; 56: 116-7.
4. Littlewood JM, Wolfe SP, Conway SP. Diagnosis and treatment of intestinal malabsorption in cystic fibrosis. Pediatr Pulmonol 2006; 41: 35-49.
5. Rovner AJ, Schall JI, Mondick JT, et al. Delayed Small Bowel Transit in Children With Cystic Fibrosis and Pancreatic Insufficiency. J Pediatr Gastroenterol Nutr 2013; 57: 81-4.
6. Mascarenhas MR, Mondick J, Barrett JS, et al. Malabsorption Blood Test: Assessing Fat Absorption in Patients With Cystic Fibrosis and Pancreatic Insufficiency. J Clin Pharmacol 2015; 55: 854-65.
7. Woestenenk JW, Van der Ent CK, Houwen RHJ. Pancreatic Enzyme

Replacement Therapy and Coefficient of Fat Absorption in Children and Adolescents With Cystic Fibrosis. J Pediatr Gastroenterol Nutr 2015; 61: 355–60.

8. Borowitz D, Konstan MW, O'Rourke A et al. Coefficients of fat and nitrogen absorption in healthy subjects and individuals with cystic fibrosis. J Pediatr Pharmacol Ther. 2007; 12: 47-52.
9. Taylor CJ, Thieroff-Ekerdt R, Shiff S, et al. Comparison of two pancreatic enzyme products for exocrine insufficiency in patients with cystic fibrosis. J Cyst Fibros 2016; 15: 675-80.
10. Turck D, Braegger CP, Colombo C, et al. ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. Clin Nutr 2016; 35: 557-77
11. Sinessappel M, Stern M, Littlewood J, et al. Nutrition in patients with cystic fibrosis: a European Consensus. J Cyst Fibros 2002; 1: 51-75.
12. Gelfond D, Borowitz D. Gasotrintestinal complications of Cystic Fibrosis. Clin gastroenterol hepatol 2013; 11: 333-42
13. Santos V, Cardoso AV, Lopes C, et al. Cystic fibrosis -Comparison between patients in paediatric and adult age. Rev Port Pneumol 2017; 23: 17-21.
14. Sikkens ECM, Cahen DL, Kuipers EJ, et al. Pancreatic enzyme replacement therapy in chronic pancreatitis. Best Pract Res Clin Gastroenterol 2010; 24: 337-47.
15. Tardelli ACS, Camargos PAM, Penna FJ, et al. Comparison of Diagnostic Methods for Pancreatic Insufficiency in Infants With Cystic Fibrosis. J Pediatr Gastroenterol Nutr 2013; 56: 178-181.
16. Williams N, Moriatis M, Chambers GM, et al. The role, yield and cost of paediatric faecal elastase-1 testing. Pancreatology 2016; 16: 551-4.
17. Schall, JI, Bentley T, Stallings V. Meal patterns, Dietary Fat Intake and pancreatic enzyme use in preadolescent children with Cystic Fibrosis. J Pediatr Gastroenterol Nutr 2006; 43: 651-9.

18. Brady MS, Garson JL, Krug SK, et al. An enteric-coated high-buffered pancrelipase reduces steatorrhea in patients with cystic fibrosis: a prospective, randomized study. J Am Diet Assoc 2006; 106: 1181-6.
19. Borowitz D, Gelfond D, Maguiness K, et al. Maximal daily dose of pancreatic enzyme replacement therapy in infants with cystic fibrosis: A reconsideration. J Cyst Fibros 2013; 12: 784-5.

PAPER 3

NUTRITIONAL STATUS, NUTRIENT INTAKE AND USE OF ENZYME SUPPLEMENTS IN PAEDIATRIC PATIENTS WITH CYSTIC FIBROSIS; A EUROPEAN MULTICENTRE STUDY WITH REFERENCE TO CURRENT GUIDELINES

Joaquim Calvo-Lerma[1*], Jessie M. Hulst[2], Inês Asseiceira[3], Ine Claes[4], Maria Garriga[5], Carla Colombo[6], Victoria Fornés[1], Sandra Woodcock[2], Tiago Martins[3], Mieke Boon[4], Mar Ruperto[5], Sylvia Walet[2], Chiara Speziali[6], Peter Witters[4], Etna Masip[1], Celeste Barreto[3], Kris de Boeck[4] and Carmen Ribes-Koninckx[1] on behalf of MyCyFAPP Project.

1 Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell, 106. Torre A, planta 0. 46026 Valencia, Spain

2 Erasmus Medical Center, Sophia Children's Hospital, Rotterdam, the Netherlands.

3 Associação para a Investigação e Desenvolvimento da Faculdade de Medicina, Avenida Professor Egas Moniz, 1649-028 Lisboa

4 Pediatric Pulmonology and Cystic Fibrosis Unit, Department of Pediatrics, University Hospitals Leuven, Belgium

5 Hospital Universitario Ramón y Cajal. Carretera Colmenar Viejo km 9, 100. 28034 Madrid, Spain.

6 CF Center, Università degli Studi di Milano.Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Via Commenda 9, 20122 Milan (Italy)

* Corresponding autor

ABSTRACT

Background: The New European guidelines have established the most updated recommendations on nutrition and pancreatic enzyme replacement therapy (PERT) in CF. In the context of MyCyFAPP project - a European study in children with CF aimed at developing specific tools for improvement of self-management - the objective of the current study was to assess nutritional status, daily energy and macronutrient intake, and PERT dosing with reference to these new guidelines.

Methods: Cross sectional study in paediatric patients with CF from 6 European centres. SD-scores for weight-for-age (WFA), height-for-age (HFA) and body mass index-for-age

(BMI) were obtained. Through a specific 4-day food and enzyme-dose record, energy and macronutrients intake and PERT-use (LU/g lipids) were automatically calculated by the MyCyFAPP system. Comparisons were made using linear regression models.

Results: The lowest quartiles for BMI and HFA were between 0 and -1SD in all the centres with no significant differences, and 33.5% of the patients had a SD-score < 0 for all three parameters. The minimum energy intake recommendation was not reached by 40% of the children and mean nutrients intake values were 14%, 51% and 34% of the total energy for protein, carbohydrates and lipids respectively. When assessed per centre, reported PERT doses were in the recommended range in only 13.8% to 46.6% of the patients; from 5.6% up to 82.7% of children were above the recommended doses and 3.3% to 75% were below.

Conclusion: Among the 6 centres, a large variability and inconsistency with new guidelines on nutrition and PERT-use was found. Our findings document the lack of a general criterion to adjust PERT and suggest the potential benefit of educational and self-managerial tools to ensure adherence to therapies, both for clinical staff and families. They will be taken into account when developing these new tools during the next stages of MyCyFAPP Project.

KEYWORDS: Cystic Fibrosis, nutritional status, pancreatic insufficiency, PERT, nutritional requirements macronutrients, guidelines, paediatrics, self-management, telemedicine

1. INTRODUCTION

Antibiotics, physiotherapy and nutrition are considered the three pillars in the treatment of Cystic Fibrosis (CF) according to the most recent standards of care [1]. Whilst therapies for lung disease have been a continuous focus of research in recent years [2] less high-impact studies have addressed the improvement of the nutritional and pancreas-related aspects.

The importance of a good nutritional status has clearly emerged over the last few decades with its direct relationship to a better lung function and consequently a better overall prognosis and survival [1,3]. Obtaining and keeping a good nutritional status is, however, a challenge in most patients with CF. This has several reasons, i.e. the increased energy requirements secondary to chronic inflammation and pancreatic insufficiency (PI) (present in almost 80% of patients) on one side and the decreased energy intake and loss of nutrients due to maldigestion and malabsorption on the other side [4].

Therefore, the main nutritional goal in children with CF is to avoid inadequate nutrient intake and to follow a normal growth pattern according to age. Nutritional follow-up and intervention is essential and should aim at achieving an optimal adjustment of pancreatic enzyme replacement therapy (PERT) to correct pancreatic insufficiency as well as at prescribing a balanced diet according to nutritional needs [5,6]. The recently published guidelines on nutrition care for infants, children and adults with CF compile the most recent scientific evidence and establish recommendations regarding nutritional goals and PERT dosage [7].

In comparison with antibiotics or physiotherapy treatments, nutrition and PERT are more likely to be mainly managed by the patients or parents themselves, outside the care of the CF team. Eating and drinking is a daily need and patients are supposed to comply with a balanced diet and adjust the dose of enzymes accordingly. However, if an incorrect nutritional behaviour or an inadequate enzyme dosage occurs, it will most likely not be detected and corrected until the next contact or visit to the CF centre. Consequently, one of the ultimate goals of the medical care in CF – achieving and maintaining an adequate nutritional status – will be jeopardised rather than potentiated.

Therefore, nutritional education and treatment in CF can be considered as one of the ideal targets of mobile health (mHealth) and patients' self-management. These concepts have been pointed out as main priorities in the current European Union's

research and innovation programme, Horizon 2020. They strongly suggest that the current and future lines of medical investigation should be focused on this area [8].

In this scenario, MyCyFAPP Project (www.mycyfapp.eu) came to fruition as an innovative approach towards the self-management of nutrition and PERT in paediatric patients with CF by means of a mobile APP. The whole project includes various studies in order to generate educational resources and supporting tools addressed to patients, to ultimately achieve the overall goal.

The present study was carried out to obtain baseline information about nutrient and PERT intake and identify possible discrepancies with the current recommendations. This information will help to develop CF-specific, tailored evidence-based tools to allow for the self-management of nutrition and PERT in order to facilitate adherence to current recommendations. We therefore assessed nutritional status, daily energy and macronutrient intake, and enzyme dosing of a European population of children with CF and related these findings to the new ESPGHAN/ECFS/ESPEN nutritional guidelines [7].

2. METHODS

2.1. Subjects and Study design

Patients with CF considered for enrolment in this cross-sectional study were regularly followed in the 6 MyCyFAPP participating European CF Centres: Lisbon (Portugal), Madrid (Spain), Valencia (Spain), Milan (Italy), Leuven (Belgium) and Rotterdam (The Netherlands). Inclusion criteria required a confirmed diagnosis of CF for at least 6 months and age between 1.0 to 17.9 years. Patients who had undergone organ transplantation were excluded. Presence of pancreatic sufficiency (PS) was not considered an exclusion criterion.

The anthropometric measurements were taken and clinical data registered. Prospectively, participants completed a newly developed 4-day food and pancreatic enzymes record. The collected information was securely stored in the online MyCyFAPP project system, allowing for data storage and automatic calculations. Participants

followed their usual diet and took their regular enzyme dose as prescribed by the specialists in their centres.

The study protocol was approved by the ethical committee of each CF centre and conducted according to the Declaration of Helsinki guidelines. All parents and patients were informed about the purpose and ultimate aim of the study and were asked to sign the informed consent.

2.2. Clinical and anthropometric data

The anthropometric data were obtained by trained personnel of the CF centres. After calibration of the equipment, weight was measured using a digital scale to the nearest 0.1 kg and height was obtained with either a measuring board (supine length) or stadiometer (standing height). Body mass index was calculated as weight (kg) / height (m^2). Anthropometric measurements were converted to standard deviations scores e.g. “height for age” (HFA), “weight for age” (WFA), and “body mass index for age” (BMI) using the CDC references [9].

2.3. Nutritional data collection and calculation

The specific 4 days food and enzymes record form (FER) was developed, based on current recommendation that a 3 to 5 day diet record is necessary for a quantitative evaluation of nutrient intake [7]. The FER was structured in six sections: 3 main meals (breakfast, lunch and dinner) and 3 snacks (morning, afternoon and night). Patients were asked to indicate the exact time of each meal, the name of the dish, ingredients/food products it contained and the amount taken, as well as the PERT dose. The FER also included a household measure table with equivalences in grams and an example of a completed food record, both in order to help participants to register everything the most accurate way. The food record is the regular tool to assess dietary intake in all the centres, so all the patients were familiar with its use. Regarding the specific FER for this study, dieticians were the personnel in charge of explaining it to the patients and their families according to a common consensus criterion among the

centres. Patients were advised to follow their normal diet and to take their regular enzyme doses.

The nutritional composition databases used for the energy and nutrient intake calculation were purchased from EuroFIR® (Spain, Italy, Portugal, and Netherlands), and Nubel® (Belgium), since they were country specific and contained the nutritional facts of the particular food products in each region. Calculations were automatically performed by the system through the specifically developed calculation tools. The total daily energy intake was calculated and expressed as kcal/kg/day. Macronutrient intake was calculated in grams of lipids, carbohydrates and protein per day and expressed as percentage of total daily energy intake. The dosage of PERT was evaluated by considering the Enzyme/Substrate ratio (E/S) as the unit of dosage quantification, which was calculated as lipase units (LU) per gram of dietary fat (g fat) per meal (LU/ g fat/ meal). Additionally, the dose was calculated in terms of LU per kilogram of body weight per day (LU/kg/day) and reported as mean daily intake (LU/day).

2.4. Comparison to current recommendations

All data were compared according to the recommendations of the new nutritional guidelines for CF for nutritional status, energy and macronutrient intake and PERT dose [7]. The recommended value for the nutritional status indicators HFA, WFA and BMI SD-scores is 0 SD, which corresponds to the mean value of ~~an~~ age-matched healthy population. The current recommendation for total energy intake is 110-200% of the recommended energy intake of healthy age-matched population. For macronutrient intake the recommendations are 20% of total daily energy intake from protein, 35-40% from lipids, and 40-45% from carbohydrates. Recommended PERT dose is between 2.000 and 4.000 LU/g of dietary fat and below 10.000 LU/kg/day.

Analysis of energy intake considered the mean energy intake minus the lower recommended limit (110%) to evaluate if patients were at least fulfilling the lowest recommendation: 0 means the recommendation is achieved whilst negative values mean the patient has achieved a lower intake than the recommended.

In order to classify patients according to the enzyme dosage recommendations, three groups were established according to their mean daily PERT intake: below the recommended range (<2.000 LU/g), within the recommended range (2.000-4.000 LU/g) and above the recommended range (>4.000 LU/g). Intra-patient variability in the E/S ratio intake was assessed; this value expresses the spectrum of enzyme dosage for a certain amount of fat used by the patient at a concrete mealtime along the 4 days record.

2.5. Statistical analyses

The sample size was estimated using Monte Carlo simulations assuming normally distributed variables and aiming for a precision of ± 10% for each variable. The estimations for performing the simulations were based on data from Schall, Bentley and Stallings (2006) [6].

The data were summarized using mean (standard deviation) or median (interquartile range) in the case of continuous variables and with relative and absolute frequencies in the case of categorical variables. The association between nutritional status, age, pancreatic insufficiency, diagnosis through neonatal screening (NBS) and time lapsed from diagnosis till the present study was assessed using linear mixed models, adding the centre as a random effect. The Pearson correlation coefficient was calculated to assess age effect on SD-scores of BMI, HFA and WFA. Beta regression mixed models were used for the nutrient intake analyses. For the analyses on PERT-dosage, for each patient (4 days each, 6 meals/day) the mean daily dosage was calculated together with the standard deviation of the mean per patient representing the intra-patient variability. A p value ≤0.05 was considered statistically significant. 95% confidence intervals (95% CI) were provided for all estimates. The statistics' analyses were performed using R software (version 3.3.2).

3. RESULTS

A total of 207 paediatric patients with CF from 6 different participating European CF centres were enrolled. **Table 4.5** shows the patients' characteristics according to centre. There was a slight predominance of male patients (53.3%). Only 33% of the patients had been diagnosed through new born screening (NBS), with remarkable differences among centres. Ninety-one per cent of the patients suffered from PI.

Table 4.5. Clinical and demographic characteristics of the study cohort

	Lisbon (n=30)	Madrid (n=33)	Valencia (n=36)	Milan (n=30)	Leuven (n=29)	Rotterdam (n=49)	TOTAL (n=207)
Mean age, years (SD)	10.6 (4.8)	7.6 (5.9)	8.4 (5.1)	7.9 (5.5)	8.2 (5.7)	7.1 (4.0)	8.3 (1.2)
Male % (n)	43.3 (13)	60.6 (20)	50 (18)	63.3 (19)	62.1 (18)	46.2 (24)	53.3 (112)
Newborn screening, % (n)	0 (0)	72.7 (24)	13.9 (5)	90.0 (27)	3.4 (1)	23.1 (12)	32.9 (69)
Mean age at diagnosis (years) (SD)	2.5 (3.0)	0.1 (0.2)	1.3 (2.0)	0.4 (1.8)	0.6 (1.0)	1.0 (2.5)	1.1 (1.0)
Pancreatic insufficiency % (n)	90.0 (27)	81.8 (27)	100 (36)	100 (30)	100 (29)	80.8 (42)	91.0 (191)

SD, standard deviation

3.1. Nutritional status

Figure 4.5 shows the median values of anthropometric parameters (BMI, WFA, HFA in SD-scores) and their relative positions as compared to age-matched healthy population (0 SD) for the different centres. First quartiles for BMI and HFA, (i.e. 25% of patients) were between 0 and -1SD except from one centre (Valencia, BMI), and third

quartiles (i.e. 75% of children) for the three indicators were comprised between 0 and +1 SD in all centres (except for Milan, WFA). Some patients (33.5%) had a SD-score < 0 for the three parameters, but only 4 patients had SD values < -2 SD for all of them. No correlations were found between any of the parameters (BMI, HFA or WFA) and age and no statistically significant differences were found among the centres for any of the parameters.

Being diagnosed by NBS, was not associated with a significantly better nutritional status outcome, in terms of BMI, WFA and HFA, when considering both age of diagnosis and time since diagnosis. The nutritional status of patients with PS was similar to that of the children with PI in terms of mean SD-scores (mean SD-scores for HFA p=0.65, 95% CI [-0.353, 0.537]), WFA p=0.43, 95% CI [-0.246, 0.587]) and BMI p=0.44 [-0.239, 0.564]).

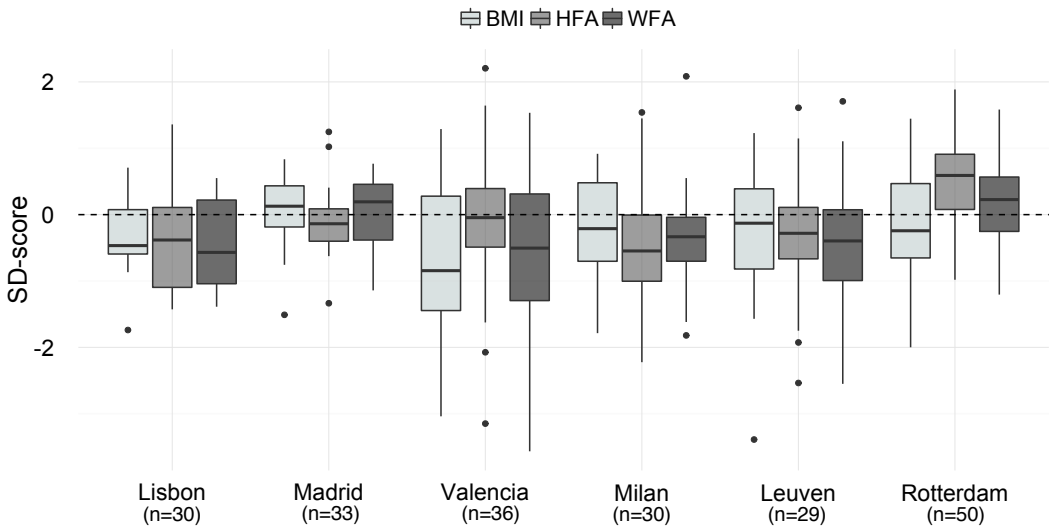


Figure 4.5. Boxplots of anthropometric parameters in the different CF centres: BMI SD-scores, HFA SD-scores and WFA SD-scores. BMI: body mass index for age; HFA: height for age; WFA: weight for age

3.2. Energy and Nutrients intake

Overall, the mean daily protein intake varied from 10% to 17% of the total daily energy intake with a mean consumption of 14% (**Figure 4.6A)**, not reaching the

recommendation of 20%. The mean value of carbohydrate intake was 51% (**Figure 4.6B**), with mean values for all centres above the recommended range. Altogether, the mean lipid intake of all centres was close to the lower threshold (34%) and ranging between 27-38% of the total daily energy intake. Only 3 centres fulfilled the recommendation for lipids (**Figure 4.6C**).

Overall, the recommended lower limit of daily energy intake was not reached by 46% of the patients. **Figure 4.6D** represents the mean value and 95% confidence interval of the daily energy intake per centre as compared to the lowest recommendations. For all the centres the mean value was slightly higher than the recommendation (+50kcal). However, the mean energy intake in two centres, those with the highest carbohydrates consumption and the lowest lipids consumption, was found to be 20-200kcal lower than the recommended intake.

Age was significantly associated with the percentage of nutrients' intake in all the centres: mean carbohydrate consumption decreased with age ($p<0.001$) by 1.1 to 2.5% per year, while mean lipid consumption increased ($p<0.001$) 0.8 to 2.2% a year and protein 0.7-1.8%. There were no differences between PS and PI patients regarding nutrient or energy intake.

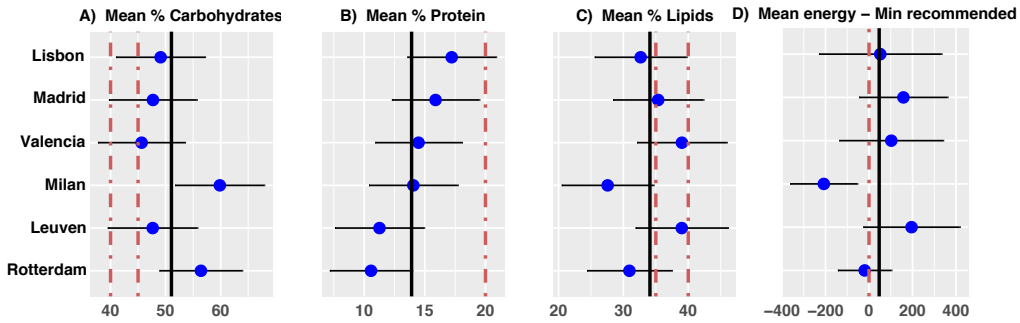


Figure 4.6. Mean values (with 95% confidence interval) of macronutrients intake as percentage of total daily energy intake: carbohydrates (2A), protein (2B) and lipids (2C). Vertical dotted lines represent the recommended intake range or value; vertical line represents the mean value of all the centres. Total daily energy intake (2D) represented as mean daily energy intake per patient minus the minimum amount recommended (110% from the recommendation in an age-matched healthy population).

3.3. Use of PERT

The mean PERT dose (LU/g fat/meal) differed widely between meals and centres. **Table 4.6** shows that the majority of patients in all centres either exceeded or did not reach the advised range: up to 82.7% of patients in one centre took more enzymes than recommended, while in other centres up to 75% of patients had a PERT dose lower than 2.000 LU/g fat/meal. The percentage of patients fulfilling the recommended dose ranged between 13.8 and 46.6%. Considering the dose of PERT as referred to kg of body weight, patients in one centre exceeded the maximum recommended limit of 10.000 LU/kg/day, with a mean (SD) intake of 16.641 (10.568) LU/kg/day. The doses for the centres below the recommendations spread from 3.200 (1.871) LU/kg/day to 8.715 (2.199) LU/kg/day.

Table 4.6. Doses of PERT in the study cohort

	Lisbon (n=30)	**Madrid (n=33)**	**Valencia (n=36)**	**Milan (n=30)**	**Leuven (n=29)**	**Rotterdam (n=49)**
Mean (SD) LU/kg/day	3200 (1871)	5795 (1313)	3206 (2346)	8715 (2199)	16641 (10568)	5523 (3714)
Mean (SD) LU/g fat/meal	1562 (802)	3616 (4974)	1559 (1347)	4362 (1477)	7858 (5027)	4209 (5482)
Mean (SD) LU/day	94081 (60313)	189750 (105476)	86364 (65386)	236375 (159745)	394655 (230324)	135306 (88090)
Enzyme supplements preparation (LU)	1.000* 5.000** 10.000	5.000** 10.000 25.000	1.000* 5.000** 10.000 25.000	10.000 25.000	10.000 25.000 40.000	5.000** 10.000 25.000
Patients with PERT dose (%) <2.000 LU/g of lipids	70	42.4	75	3.3	3.5	24

	Lisbon (n=30)	Madrid (n=33)	Valencia (n=36)	Milan (n=30)	Leuven (n=29)	Rotterdam (n=49)
Patients with PERT dose (%) 2000 – 4000 LU/g of lipids	30	14.4	19.4	46.6	13.8	42
Patients with PERT dose <4000 LU/g of lipids	0	15.2	5.6	50	82.7	34

PERT pancreatic enzyme replacement therapy; *SD* standard deviation; *LU* lipase units

*Capsules prepared in the Pharmacy Unit of the hospital, especially for children <1-2 years.

** Pancreatin granules (1 measuring scoop = 5.000 LU)

Overall, when considering main meals (breakfast, lunch, dinner) as compared to snacks (**Figure 4.7a**), enzyme doses or E/S (LU/g lipids) had a similar pattern, i.e. doses at snacks were not higher than at main meals or vice-versa. Considering different meals individually, there was not a common trend or pattern in enzyme doses, neither among patients from the same centre, or when comparing different centres. However, for some meals, mainly snacks, no enzymes were taken even though the meal contained lipids (data not shown in **Figure 4.7a** since minimum value represented is 250 LU/g of lipids). The upper outliers of E/S values were found at snack meals in all centres.

Age was not associated with PERT dosage at any centre except in Valencia, where the oldest children had the highest E/S values ($p<0.01$) and in Rotterdam where, on the contrary, the oldest patients had the lowest E/S ($p<0.01$).

Large differences in E/S ratios among meals for the same patient (intra-patient variability) were obtained, ranging from mean values of 400 LU/g of fat in some meal types to 3200LU/g of fat in others (**Figure 4.7b**). No concrete meal type was significantly associated with a higher or a lower intra-patient variability in E/S, but snacks meals were the only ones showing cases of 0 variability. Centres with lower E/S ratios had the

lowest intra-patient variability. Finally, age was not associated with intra-patient variability in E/S (p->0.05) in all the centres.

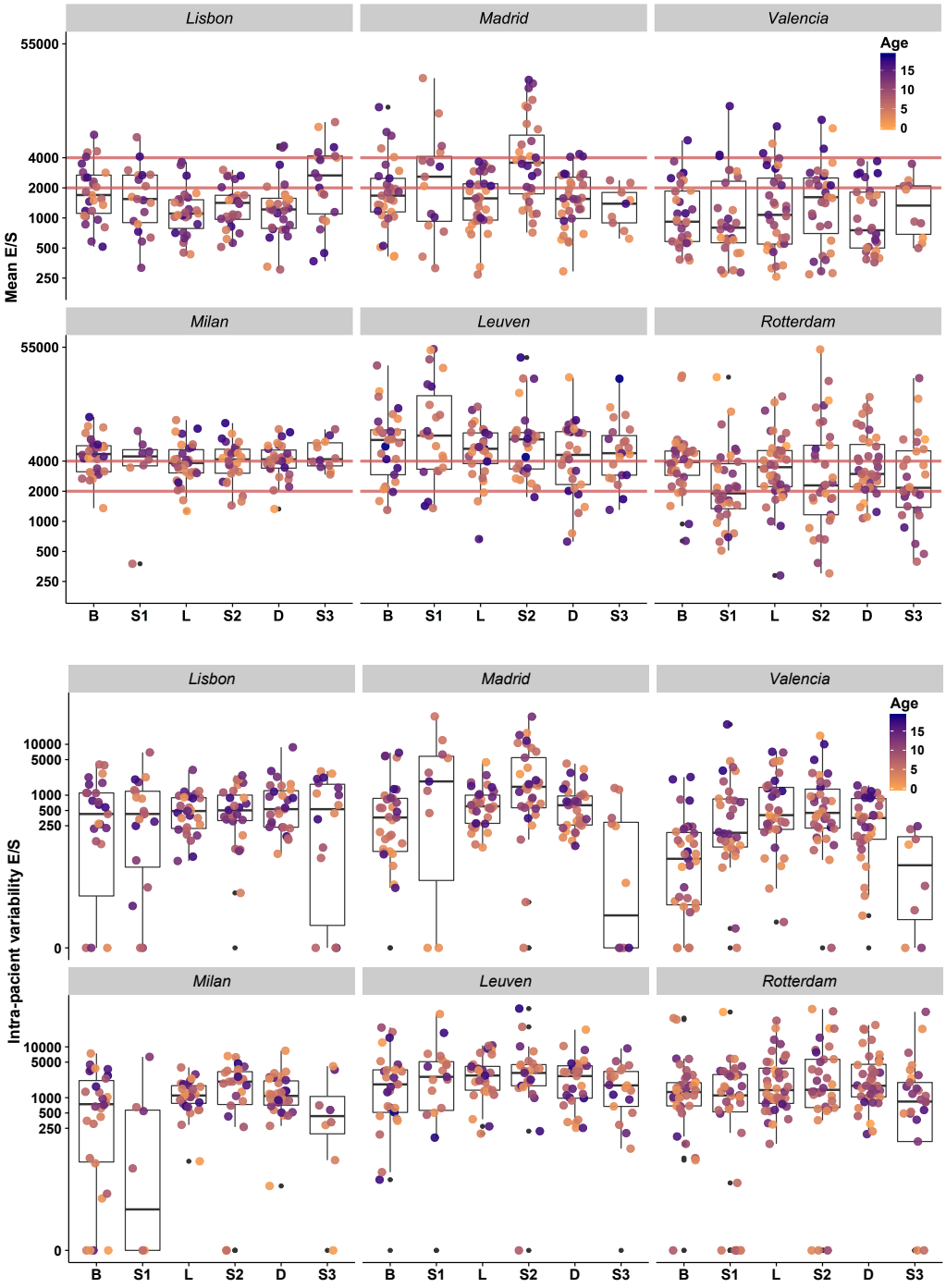


Figure 4.7. Dose of PERT per meal type and centre. Expressed as mean (E/S in LU/g fat) in the upper part and expressed as intra-patient variability (SD E/S) in the bottom part. B, breakfast; S1, snack 1; L, lunch; S2, snack 2; D, dinner; S3, snack 3. Horizontal lines indicate the recommended range for dose in terms of lipase units per gram of fat (2000-4000 LU/g fat) according to Turck et al. 2016.

4. DISCUSSION

Through the present study we have characterized nutritional status, energy and nutrients intake and meal-specific use of PERT of a European group of paediatric patients with CF compared to what is recommended by the new guidelines on nutrition in CF [7].

Firstly, we have found a very wide range of intra-centre variability in terms of SD-scores for BMI, height and weight for age, with the majority of the results standing between 0 and -1SD. Severe malnutrition (values < -2SD [7]) indicators were, however, found only occasionally. Association between diagnosis by NBS and better outcomes of nutritional status, as previously reported in wider series [1,4], was not found in our study population with only 33% diagnosed by NBS.

Secondly, the study has pictured centre-specific macronutrients distribution and energy intakes. In general terms, protein intake was lower than recommended in all centres, none of which achieved the daily intake objectives; this fact could possibly be explained by CF teams still applying the previous recommendations [10], although not completely compliant with these neither.

With regard to lipids, some centres more successfully accomplished recommendations than others, probably due to the higher amounts of oil and fats consumed related to the local nutritional habits. Of note, centres with the highest carbohydrate intake had the lowest lipids and energy intake and vice-versa. Our results are comparable to those of a previous multicentre study conducted in the US in infants and toddlers with CF, that showed an intake of 33.8% of energy from fat, 55.8% from carbohydrates and 12.4% from protein, and only 50% achieving the minimum energy recommendation [11]. However, the mean energy intake of the whole group was slightly

above the 110% limit, evidencing that despite almost half of the patients not complying with it, they were very close to the recommendation. This is clearly reflected in Figure 2D: there is one single centre contributing to the low overall value, while the mean energy intake in four centres is higher than the mean of the group and than the minimum recommended. In another study carried out in the US, 86 subjects aged 6.0-8.9 years had around 37% of fat intake and the 120% energy goal was not reached by 61% of the children [6]. In addition, in a larger population of 234 Dutch children aged 0-18 years fat intake represented 34-36 % of total energy, which in turn was 88 to 119% of recommendations for an healthy age-matched population [12].

Finally, our results have evidenced the lack of uniformity in PERT dosage among the centres, E/S ratios ranging from mean values of 1520 up to 7758 LU/g fat/meal with considerable differences among centres. In terms of maximum recommended daily dose, only in one centre patients exceeded the maximum recommended dose of 10000 LU/kg/day whilst in others the mean value was far below. Despite this fact, no cases of fibrosing colonopathy were reported in any of the centres. This is in accordance to a previous study showing that 10000 LU/kg/day should not be any longer a restriction[13], although the new guidelines maintain this figure as the maximum recommended. We believe the outstripping result in one centre is related to the enzyme supplements preparation of 40.000 LU recently instituted. Likewise, the formulation of 1.000 LU available in the centres with the lowest PERT doses may be the reason for this finding. The mean value of the whole group (7.144 LU/kg/day) was, however, very similar to that reported in a Dutch population (7.627LU/kg/ day) [4].

A comprehensive study in children 8-11 years assessing adherence to PERT concluded that 88% of the patients were within the previously recommended range of 500-4000LU/g fat and only 4% were below [6]. According to our results, in some centres very high percentage of patients were below the new recommended PERT dosage range (<2000 LU/g), but if we take into account the 2002 guidelines (lower limit of 500 LU/g fat), we would find a similar percentage of patients under the lower limit as the previous study [6]. We highlight the fact that despite the very wide range of PERT intake

(overdosing vs. underdosing) between centres, no differences were found in terms of nutritional status. There was no association either between PERT dose and energy intake. On the other hand, it is well known that there is an enormous variability in the response to PERT among patients; in a large retrospective study in children with CF no correlation was found between enzyme dose and the degree of fat malabsorption [13]. It is also possible that differences would have been found when gastrointestinal complaints were taken into account.

The discrepancies in PERT dosing criteria found in our study reinforces the absence at the present time, of a well-established and evidence-based adjustment method. The authors of the new guidelines acknowledge this insufficient evidence for the recommended PERT dosage, and some studies have highlighted the need of conducting research in order to improve PERT adjustment [14,15]. Other authors also highlight the need to investigate individual factors, such as gastric emptying time or intestinal transit time, and inherent-to-food factors, like physicochemical characteristics of the food matrix or the type of fat, as determinants on the efficiency of the pancreatic enzymes supplements [16-19].

Overall, the results of this study could have an impact on daily clinical practice in terms of nutritional advice and education to parents. We observed a change in macronutrients distribution with age in all centres. The younger the patient, the higher the carbohydrates intake (exceeding the daily recommendations), but as children got older, the percentage of energy intake from carbohydrates decreased in favour of an increase in that from protein and lipids. A similar tendency was previously reported in a cohort of Spanish patients by Calvo-Lerma et al 2015 (PO-AHP-0006 ESPGHAN 48th meeting). Higher carbohydrates intake and lower protein as compared to the recommendations was also observed in another study with a population aged 7-35 months–[11]. Thus intervention to promote adequate dietary patterns should start at early age.

Moreover, the high intra-patient variability for specific meals and regarding the enzyme to substrate dosage implicates that patients took a fixed enzyme dose for each

particular meal independently of what they ate. For example, if the child takes a fixed amount of 2 enzyme capsules for breakfast each day, but the fat intake varies between 10-20 g of fat, the E/S ratio would be 2.000 LU/g of fat (inside the recommended range) – 1.000 LU/g (below the recommended amount). This would mean an intra-patient variability of 1000 LU/g of fat between two days. These findings suggest that patients should be aware and take into account the content of fat in every meal in order to adjust the dose accordingly, with the target of reaching the optimal/recommended E/S ratio. For this purpose, educational material and adequate tools should be available for patients at any time to help them to decide the dose of enzymes they should take for a particular meal.

In this regard the results obtained, in the context of MyCyFAPP Project, may be very useful. The identification of the specific nutritional imbalances to be addressed and the goals established in the new guidelines will be considered as the project's premises to start building up the tailored educational resources and tools that eventually will be in the hand of the patient with the mobile APP.

The major strength of the study is the use of robust methodology that makes it of scientific value, including specific and detailed food and enzyme information, reliable databases and tools to calculate the nutritional facts, the common consensus practice in explaining patients about how to fill in the record, expert dieticians participating in the study design and its implementation, and the nature of the statistical analyses performed (application of models rather than tests). Additionally, this is the first time, to our knowledge, that a study has been conducted that specifically compares the current practice to the newly published experts' guidelines in a multicentre way in 5 different European countries.

Our study has some limitations. Despite five European countries (6 different centres) from different areas were enrolled - two northern European cities (Leuven and Rotterdam) and four from the south (Lisbon, Madrid, Valencia and Milan) – the conclusions may not apply to all European countries. Indeed, we have found that dietetic and therapeutic patterns vary from one region to another, thus future studies

could consider the inclusion of other countries. Although the patients completed the dietary data in the food record form in detail, only a reduced number filled in the specific questions about gastrointestinal symptoms and stools frequency, so those data could not be used for the analyses. Besides, it cannot be ruled out that dietary data recorded was totally accurate, since filling in the food record might have supposed an extra burden to the patients. In the future, MyCyFAPP will provide a guided and user-friendly food recording system on a mobile APP. This will improve reliability and precision on the collected data. Another limitation of the study could be the number of patients included; however, from the descriptive analysis' perspective, which was the primary objective, the sample size was adequate. Besides other studies in the field included a similar or smaller number of patients [6,11,12].

We found no association between NBS diagnosis and better nutritional outcomes - as previously reported– probably due to the small sample of patients applying for such analysis, and to the random nature of the inclusion criteria, only 32.8% of patients having been diagnosed by NBS. Moreover, the mean interval between NBS until the present time was short (only 4 years), and similarly mean age was low, while generally benefits from very early diagnosis and intervention is more evident with age increase [19]. Being pancreatic sufficient was also not associated with a better nutritional status, but this sample represented only 9% of the total study population. We did not study the association between nutritional status and FEV1 as it was scheduled with different periodicity in the different centres, and as this was a cross-sectional study, the data on FEV1 were insufficient to apply the comparative analysis.

Our project is further studying the dietary origin of the nutrients in terms of foods sources in the different countries. This knowledge will allow for advising patients in a more practical way i.e. how to adjust the intake of certain products in order to correct the concrete nutritional imbalances, like a deficient intake of lipids. Additional information about type of lipids consumption in terms of degree of fatty acids' saturation could be relevant since variations in the type of fatty acids have been

associated with different digestion fates [20]; this could affect PERT efficiency and thus dosing needs. Finally, results have reinforced the need to focus the research on an evidence-based method for PERT, which is being addressed in other areas of the project.

To conclude, in a paediatric European CF population we have found discrepancies between the new guidelines on nutrition along with a wide spectrum of differences both in nutrients intake and PERT among the six centres. Furthermore, our results proof the lack of a general criterion to adjust PERT and the potential benefit of educational and self-managerial tools for patients' better adherence to therapies. The development of these tools is the target of the next steps of MyCyFAPP Project.

ACKNOWLEDGEMENTS

Authors of this paper, on behalf of MyCyFAPP consortium, acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme for funding the project (ref. 643806).

REFERENCES

1. Yen EH, Ms HQ, Borowitz D. Better Nutritional Status in Early Childhood Is Associated with Improved Clinical Outcomes and Survival in Patients with Cystic Fibrosis. *J Pediatr*. 2013;162(3):530-535.e1. doi:10.1016/j.jpeds.2012.08.040.
2. Amin R, Ratjen F. Emerging drugs for cystic fibrosis. 2014;19(1):143-155.
3. Corey M, Mclaughlin FJ, Williams M, Levkon H. A COMPARISON OF SURVIVAL , GROWTH , AND PULMONARY FUNCTION IN PATIENTS WITH CYSTIC FIBROSIS IN BOSTON AND TORONTO. *J Clin Epidemiol*. 1988;41(6):583-591.
4. Woestenenk JW, Ent CK Van Der, Houwen RHJ. Pancreatic Enzyme Replacement Therapy and Coefficient Cystic Fibrosis. *Pancreatology*. 2015;61(3):355-360. doi:10.1097/MPG.0000000000000784.
5. Haupt ME, Kwasny MJ, Schechter MS, Mccolley SA. Pancreatic Enzyme Replacement Therapy Dosing and Nutritional Outcomes in Children with Cystic

Fibrosis. *J Pediatr*. 2014;164(5):1110-1115.e1. doi:10.1016/j.jpeds.2014.01.022.

6. Schall, JI, Bentley, T, Stallings V. Meal patterns, Dietary Fat Intake and pancreatic enzyme use in preadolescent children with Cystic Fibrosis. *J Pediatr Gastroenterol Nutr*. 2006;43(November):651-659.
7. Turck D, Braegger CP, Colombo C, et al. ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clin Nutr*. 2016;PublishAh. doi:10.1016/j.clnu.2016.03.004.
8. Horizon 2020. Work Programme 2014-2015. Health, Demographic change and wellbeing. European Commission Decision C (2015) 2453 of 17 April 2015.
9. Kuczmarski R, Ogden C, Guo S, et al. *2000 CDC Growth Charts for the United States : Methods and Development*. Vital Health Stat 11
10. Sinaasappel M, Stern M, Littlewood J, Wolfe S, Steinkamp G, Heijerman HGM. Nutrition in patients with cystic fibrosis : a European Consensus □. 2002;1:51-75.
11. Powers SW, Patton SR, Byars KC, et al. Caloric Intake and Eating Behavior in Infants and Toddlers With Cystic Fibrosis. *Pediatrics*. 2002;109(e75). doi:10.1542/peds.109.5.e75.
12. Woestenenk JW, Castelijns SJAM, Ent CK Van Der, Houwen RHJ. Dietary intake in children and adolescents with cystic fi brosis. *Clin Nutr*. 2014;33(3):528-532. doi:10.1016/j.clnu.2013.07.011.
13. Woestenenk JW, Ent CK Van Der, Houwen RHJ. Pancreatic Enzyme Replacement Therapy and Coefficient Cystic Fibrosis. 2015;61(3):355-360. doi:10.1097/MPG.0000000000000784.
14. Hu M, Li Y, Decker EA, McClements DJ. Role of calcium and calcium-binding agents on the lipase digestibility of emulsified lipids using an in vitro digestion model. *Food Hydrocoll*. 2010;24(8):719-725. doi:10.1016/j.foodhyd.2010.03.010.
15. Li Y, Hu M, McClements DJ. Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: Proposal for a standardised pH-stat

method. *Food Chem*. 2011;126(2):498-505. doi:10.1016/j.foodchem.2010.11.027.

16. Fieker A, Philpott J, Armand M. Enzyme replacement therapy for pancreatic insufficiency: Present and future. *Clin Exp Gastroenterol*. 2011;4(1):55-73. doi:10.2147/CEG.S17634.
17. Borowitz D, Gelfond D, Maguiness K, Heubi JE, Ramsey B. Maximal daily dose of pancreatic enzyme replacement therapy in infants with cystic fibrosis: A reconsideration. *J Cyst Fibros*. 2013;12(6):784-785. doi:10.1016/j.jcf.2013.05.011.
18. Rovner AJ, Schall JI, Mondick JT, Zhuang H, Mascarenhas MR. Delayed small bowel transit in children with cystic fibrosis and pancreatic insufficiency: Commentary. *Inflamm Bowel Dis Monit*. 2014;14(2):65-66. doi:10.1097/MPG.0b013e318290d112.
19. Farrell PM, Kosorok MR, Laxova A, et al. Nutritional beneffits of neonatal screening for cystic fibrosis. *New Engl J Med tional*. 1997;337(14):963-969.
20. Hunter EJ. Studies on effects of dietary fatty acids as related to their position on triglycerides. *Lipids*. 2001;36(7):655-668. doi:10.1007/s11745-001-0770-0.

PAPER 4

CHILDREN WITH CYSTIC FIBROSIS PRESENT WITH DIETARY IMBALANCES: A EUROPEAN MULTICENTRE COMPARISON OF FOOD GROUPS AND ORIGIN OF NUTRIENT INTAKE

Joaquim Calvo-Lerma[1*] (MSc), Jessie Hulst[2] (MD, PhD), Mieke Boon[3] (MD, PhD), Tiago Martins[4] (BSc), Mar Ruperto[5] (PhD), Carla Colombo[6] (MD, PhD), Etna Masip[1] (MD), Sandra Woodcock[2] (BSc), Ine Claes[3] (BSc), Inês Asseiceira[4] (BSc), María Garriga[5] (PhD), Anna Bulfamante[6] (MSc), Victoria Fornés-Ferrer[1] (MSc), Sylvia Walet[2] (BSc), Paula Crespo[1] (PhD), Lauretta Valmarana [6] (BSc), Luisa Pereira[4] (MD, PhD), Kris de Boeck[3] (MD, PhD), and Carmen Ribes-Koninckx[1] (MD, PhD).

[1] Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell 106. 46026 Valencia (Spain). Tel. +34 961246712

[2] Erasmus Medical Center, Sophia Children's Hospital. Postbus 2040 3000 CA Rotterdam Rotterdam (The Netherlands).

[3] Department of paediatrics, University hospital of Leuven, University of Leuven. Herestraat 49, 3000 Leuven (Belgium)

[4] Associação Portuguesa para a Investigação e Desenvolvimento da Faculdade de Medicina. Av. Prof. Egas Moniz 1600-190 Lisbon (Portugal).

[5] Servicio Madrileño de Salud - Hospital Universitario Ramón y Cajal. Ctra. Colmenar Viejo Km. 9,100, 28034, Madrid (Spain).

[6] Università degli Studi di Milano. Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico. Via Commenda 9, 20122 Milan (Italy).

*Corresponding author: joaquin_calvo@iislafe.es

RESEARCH SNAPSHOT

Research question

How does food intake explain the imbalances in nutrient intake in European children with Cystic Fibrosis?

Main findings

In this cross-sectional prospective study including 207 paediatric patients with cystic fibrosis from the MyCyFAPP Project, sugar intake represented 10.8 to 27.2% of the total daily energy intake among the centres and the saturated fatty acids represented >35% from total lipid intake in all the centres. These imbalances are associated to the low

consumption of fish, legumes, fruit, vegetables and nuts and the overconsumption of sweets and snack processed products in our population.

ABSTRACT

Background: Optimal nutrition in Cystic Fibrosis (CF) improves prognosis and survival. Increased caloric intake recommendation for this population should take into account the risk of saturated fatty acids overconsumption.

Objective: to describe the nutrient intake and the food groups' intake in European paediatric CF patients in order to assess the relative contribution of each food group to total macronutrient intake.

Design: We performed a cross-sectional study. The participants recorded dietary intake. Specifically developed nutritional composition databases were used to obtain nutritional data, including macronutrients and food groups, according to previously standardised criteria.

Participants: 207 paediatric patients with CF from 6 European centres involved in MyCyFAPP project

Main outcome measures: participants reported dietary intake with a detailed 4-day food record.

Statistical analysis performed: Descriptive analysis on nutrient intake, food groups consumption and dietary origin of macronutrients were conducted with R software.

Results: Similar patterns were found in nutrient and food groups' intake as both sugar and saturated fatty acids contributed >10% each to the total daily energy intake in all the centres. Furthermore, milk, meat, sweets and snacks and oils were the main sources of lipids in all centres. On the other hand large differences were found in other nutrient and food groups' intake since sweets and snacks were consumed 1-2 times a day, and fruit and vegetables were consumed 2-3 times/day.

Conclusions: Full characterization of nutrient and food groups' intake revealed several dietary imbalances especially high sugar and saturated fatty acid intake and a high variability in patterns between centres. These results can be used to develop upcoming

educational tools and can set the basis for future tailored improvement of adherence to the nutritional recommendations.

Keywords

Cystic Fibrosis, nutrient intake, food groups, saturated fatty acids, paediatrics.

1. INTRODUCTION

Children with cystic fibrosis (CF) have a high nutritional risk due to several reasons. Pancreatic insufficiency is present in 80-90% of CF patients [1,2], and if not adequately corrected, results in maldigestion of nutrients leading secondarily to malabsorption [3], with a negative impact on nutritional status. This, summed to the increased energy requirements and possible additional lack of appetite with compromised intake, results in a negative nutrient balance and compromises the nutritional status [1]. Prevention and treatment of malnutrition is of major importance because an optimal nutritional status improves the overall prognosis and survival [3,4].

Nutritional intervention in CF patients is based on dietary recommendations of energy intake and macronutrient distribution, fat-soluble vitamin supplementation and life-long pancreatic enzyme replacement therapy (PERT). In this respect, the current European Guidelines on Nutrition in CF have recently updated the nutritional goals and dietary recommendations to be achieved in CF patients [5].

These guidelines advice the *ad-libitum* consumption of high fat foods when weight gain is necessary, although acknowledging concerns of increased saturated fats in the diet [5]. With the remarkable improvement in survival, patients with CF may become at risk of cardiovascular disease, obesity [6,7], and other age-related conditions associated to the high consumption of saturated fatty acids (SFA) and sugar. Therefore, reaching a balanced and healthy diet will become of upmost importance, and preference to fats with unsaturated fatty acids is recommended also in case of low weight [5].

Moreover, a recent systematic review that focused on the historical perspective of dietary intake studies in children with CF (1969-2016), showed that the nutrition dogma has to be extended from "nutrition for growth and survival" to "nutrition for health and wellbeing" [8]. Along the 40 evaluated studies, all considered energy intake, about 33-66% looked at macronutrient intake and only 3% included food group variety, using a 24h recall questionnaire as method in only <50%. Authors claimed for contemporary information in dietary intake in the CF population [8].

One of the main objectives of the MyCyFAPP Project [9] is to empower CF patients' self-management to achieve an optimal nutritional intake. So, in the first stage of the project, the nutritional status, actual PERT dose and macronutrient intake of a cohort of paediatric CF patients from six different European countries were compared with respect to the new guidelines on nutrition in CF [10]. We found poor compliance with the recent recommendations, especially low protein consumption, along with high carbohydrates and low lipids intake, including large differences among all the centres [10]. We acknowledged the need of assessing the nutrient and dietary intake more in detail to explain the documented imbalances and to establish specific corrective interventions in terms of choice of foods to meet the recommendations.

Therefore, the aim of the present study is to describe the nutrient intake in relation to the food groups' consumption in the children with CF in order to assess the relative contribution of each food group to total macronutrient intake. This information will help to develop CF-specific, evidence-based tools, focused on correcting imbalances and improving adherence to current CF recommendations, to allow the self-management of nutrition.

2. MATERIALS AND METHODS

2.1. Subjects and Study design

We carried out a multicentre, cross-sectional, observational study and enrolled 210 CF patients ranging in age between 2 and 17 years, in regular follow-up at 6 European CF centres: Lisbon, (Portugal, 30 patients), Madrid, (Spain, 33 patients),

Valencia (Spain 36 patients), Milan, (Italy, 30 patients), Leuven (Belgium, 29 patients) and Rotterdam (The Netherlands, 52 patients). Three patients dropped out from the study. Partial results of this study have already been published before [10].

Inclusion criteria required a confirmed diagnosis of CF since at least 6 months and age between 2 to 18 and years. Patients who had undergone organ transplantation were excluded, whereas the absence of pancreatic insufficiency (PI) was not considered an exclusion criterion.

The study protocol was approved by the Ethical Committees of each participating centre, as a sub-study within MyCyFAPP Project (Horizon 2020, 643807), and conducted according to the Declaration of Helsinki guidelines. All parents and patients >12 years gave written informed consent.

2.2. Nutritional data collection and processing

A specifically developed 4-day food record was used (**Annex 1**), where all meals were registered in terms of "name of the meal", "ingredients" and "amounts" of food. Patients were provided with written instructions, including a household measure equivalence table to support the amount of foods estimation. Dieticians were the personnel in charge of explaining it to the patients and their families according to a common consensus criterion among the centres. The methods used are described in detail in the first published paper of the MyCyFAPP project [10].

The nutritional composition databases used for the energy and nutrient intake calculation were purchased from EuroFIR® (Spain, Italy, Portugal, and Netherlands), and Nubel® (Belgium) (2015), since they were country specific and contained the nutritional facts of the particular food products in each region. Calculations were automatically performed by the system through the specifically developed calculation tools of the project. We evaluated the nutrients that were present in the majority of food items: energy, protein, carbohydrates (CH), sugar, lipids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), fibre, calcium (Ca), iron (Fe) and sodium (Na). For most of the food products, existing gaps in

sugar, SFA, MUFA, PUFA, Ca, Fe and Na were completed by consulting alternative sources, including the labelling of specific brands and other official nutritional composition databases. Information on minerals and vitamins were only scarcely available in the consulted sources and thus, were not considered. In addition, food items were assigned to a food product category or group. A total of 13 groups were established after a consensus on classification criteria was achieved among the centres: milk and dairy, meat, fish, eggs, fruit, vegetables, legumes, starchy products and grain, sweets and snacks, beverages, oils, solid fats and nuts (**Table 4.7**).

Table 4.7. Food groups and subgroups classification according to the common consensus criteria. All the foods listed in the nutritional composition databases were assigned with a food group and subgroup. Relative contribution foods to macronutrients intake was made on the basis of this classification

Group	Name of the group	Subgroup	Name of the sub-group	Examples
1	Milk & Dairy	1	Whole-fat milk	All types, including lactose-free milk
		2	Skimmed and semi-skimmed milk	All types, including lactose-free milk
		3	Yoghurt	All types, including lactose-free yoghurt
		4	Skimmed and semi-skimmed yoghurt	All types, including lactose-free yoghurt
		5	Cheese	All types
		6	Low-fat cheese	All types
		7	Milk shakes	All types
		8	Cream	All types
		9	Milk-based desserts	All types
		10	Infant formula	All types
		11	Goat milk	All types
		12	Others	All types

Group	Name of the group	Subgroup	Name of the sub-group	Examples
2	Meat	1	Chicken	
		2	Pork	
		3	Beef/bovine	
		4	Turkey	
		5	Duck	
		6	Horse	
		7	Rabbit	
		8	Lamb	
		9	Saussages/hamburgers	
		10	Cold-meat	
		11	Breaded-meat	
		12	Jar of baby food (meat-based)	
		13	Innards	
		14	Others	
3	Fish	1	White fish (low fat)	hake, monkfish (angler fish), sole, cod, sea bass
		2	Blue fish (high fat)	anchovie, sardine, mackerel, tuna, bonito, salmon, gilt-head bream, trout, bream
		3	Seafood	
		4	Canned fish in oil	
		5	Canned fish in brane	
		6	Breaded fish	
		7	Jar of baby food (fish-based)	
		8	Others	
4	Eggs	-		
5	Fruit	1	Fresh fruit	
		2	Jar of baby food (fruit-based)	
		3	Dried fruit	
		4	Fatty fruit	Avocado, etc
6	Vegetables	1	All vegetables	
		2	Jar of baby food (vegetables-based)	
		3	Vegetarian products	tofu, kefir, vegetables burger, etc
7	Legums	-		

Group	Name of the group	Subgroup	Name of the sub-group	Examples
8	Starchy products	1	Bread	
		2	Pasta	
		3	Rice	
		4	Potato	
		5	Infant cereal	
		6	Others	
9	Sweets and snacks	1	Chocolate	chocoalte bar, nutella, chocolate-based snacks like kit-kat, m&m's, etc.
		2	Sweets	candies, sweets, jellybean, lollipops, etc.
		3	Pastries and cakes	croissant, doughnut, cupcake, etc.
		4	Salty bakery	bread sticks, etc.
		5	Powder sweeteners	sugar, cocoa powder
		6	Breakfast cereal	All types: suggary, chocolate-containing, honey-containing… Cereal bars
		7	Biscuits	marie biscuit, etc.
		8	Chocolate biscuits	Chocolate-covered, chocolate-stuffed, chocolate-based, with chocolate
		9	Chips and derivates	commercial chips and similar snacks
		10	Others	For example: jam and other sugar-based or refined product
10	Beverages	1	Juices	Commercial fruit juices
		2	Soda/soft drinks	Coke, orange/lemon soda, tonic water, iced tea, lemonade, etc.
		3	Tea and coffee	tea and coffee
		4	Milk substitutes	From rice, oats, almond, soya
11	Oils	-		
12	Fats	1	Butters	
		2	Margarine	
13	Nuts	-		

2.3. Calculations and statistical analyses

For energy and nutrient intake calculations, data were grouped per patient. For the calculation of median intakes for each patient, the total intake of energy (in kilocalories, kcal) and of each nutrient (in grams, g; or milligrams, mg) was divided by 4, the total number of days the food record was filled in. Values of protein, carbohydrates, sugar and lipids were also expressed in % of the total daily energy intake. Values of SFA, MUFA and PUFA were referred to the % of the total lipid intake. The median per centre was calculated.

Frequency of consumption of food groups was calculated by dividing the total number of times a food group was consumed by each patient and divided by four (the number of recording days). Then the median per centre was calculated.

In order to calculate the dietary origin of macronutrients, the total amount (in grams) of each food group that contributed to each macronutrient intake for each patient was divided by the four recording days. Then median macronutrient intake and the percentage each food group contributed to it were calculated. Only food groups with a contribution of at least 5% to each macronutrient intake will be reported in the results section.

Data were expressed as median and interquartile range (IQR). Statistical descriptive analyses were performed using R software (version 3.3.2).

3. RESULTS

A total of 828 dietary records, each covering 24h, were obtained from 207 children with CF and 4.554 meals were described. Participants reported a median number of 5 meals (including main meals and snacks) per day. Demographic and clinical characteristics of the study population was reported before by Calvo-Lerma et al. (2017) [10].

3.1. Nutrient intake among countries

Table 4.8 shows the daily intake of macro- and micronutrients (g or mg/day) of children with CF in 6 European centres and the relative contribution of macronutrients to the total energy intake. Sugar intake was higher than 10% of the total energy intake in all the centres, with median intakes, ranging from 10.8% to 27.5%. Regarding the three types of lipids, SFA and MUFA represented the highest contribution to the total lipid intake in all the centres, both around 40%. PUFA showed the lowest representation and with large differences among centres, i.e. ranging from 9.3 to 17.6%. Dietary fibre also showed a wide spectrum of consumption from a median of 10.5 to 17.0 g/day. Concerning micronutrients intake, high differences were documented in terms of calcium and sodium. Iron was more evenly distributed among centres.

Table 4.8. Daily intake* of macro- and micronutrients of children with Cystic Fibrosis in 6 European centers

Nutrient		**Lisbon** n=30 Median (1st, 3rd Q.)	**Madrid** n=33 Median (1st, 3rd Q.)	**Valencia** n=36 Median (1st, 3rd Q.)	**Milan** n=30 Median (1st, 3rd Q.)	**Leuven** n=29 Median (1st, 3rd Q.)	**Rotterdam** n=49 Median (1st, 3rd Q.)
Protein	g/day	99.4 (86.9, 125.7)	66.0 (54.6, 90.0)	75.3 (61.9, 95.9)	59.0 (47.0, 83.9)	71.0 (54.1, 95.5)	55.4 (47.2, 71.8)
	%	18.1 (16.6, 20.7)	17.9 (16.4, 20.4)	16.1 (14.3, 18.1)	16.4 (14.5, 17.6)	14.1 (12.2, 15.2)	12.2 (10.9, 13.8)
CH	g/day	264.2 (198.9, 299.5)	143.1 (131.5, 173.4)	211.4 (165.5, 263.7)	211.0 (179.0, 255.9)	239.0 (186.2, 275.7)	249.7 (208.0, 285.8)
	%	45.0 (42.6, 47.4)	44.5 (39.9, 47.5)	44.4 (40.3, 47.9)	57.2 (50.8, 61.3)	45.7 (41.2, 49.7)	53.2 (48.8, 57.5)
Sugar	g/day	116.8 (92.5, 136.2)	78.3 (58.5, 88.7)	82.7 (69.5, 97.9)	75.7 (62.7, 102.7)	59.7 (34.5, 77.7)	129.5 (103.1, 159.4)
	%	19.6 (16.9, 22.8)	20.9 (15.1, 24.9)	16.5 (14.0, 20.0)	19.3 (15.5, 24.8)	10.8 (8.4, 12.8)	27.2 (23.8, 32.4)
Lipids	g/day	94.1 (81.2, 99.6)	64.2 (46.7, 76.2)	87.2 (73.5, 101.4)	53.6 (33.3, 74.3)	88.6 (68.9, 122.8)	65.9 (54.3, 87.0)

		Lisbon n=30 Median (1st, 3rd Q.)	**Madrid** n=33 Median (1st, 3rd Q.)	**Valencia** n=36 Median (1st, 3rd Q.)	**Milan** n=30 Median (1st, 3rd Q.)	**Leuven** n=29 Median (1st, 3rd Q.)	**Rotterdam** n=49 Median (1st, 3rd Q.)
	%	36.0 (34.6, 38.7)	37.1 (34.1, 42.2)	39.1 (34.9, 43.9)	29.5 (24.8, 34.1)	39.1 (35.5, 41.3)	33.1 (29.5, 36.7)
SFA	g/day	34.0 (26.0, 40.2)	23.05(17.1, 28.1)	31.3 (22.2, 37.5)	19.7 (13.1, 28.2)	31.6 (26.5, 45.8)	24.8 (20.1, 32.5)
	% total lipids	37.2 (34.6, 39.9)	36.2 (31.4, 38.5)	37.0 (32.6, 39.6)	37.4 (33.7, 39.7)	38.9 (31.5, 44.9)	38.6 (34.1, 41.9)
MUFA	g/day	31.0 (24.7, 35.3)	27.7 (21.6, 32.5)	33.4 (25.5, 41.2)	20.8 (13.4, 29.5)	18.9 (10.2, 27.2)	30.7 (23.0, 37.0)
	% total lipids	33.0 (30.2, 36.7)	41.4 (38.8, 46.2)	39.2 (34.5, 42.7)	41.1 (35.9, 45.4)	40.6 (36.8, 46.1)	44.5 (42.6, 47.3)
PUFA	g/day	11.8 (10.1, 16.6)	7.3 (4.6, 9.4)	10.8 (8.8, 14.6)	5.6 (3.3, 9.2)	6.5 (3.9, 10.9)	11.7 (8.9, 16.4)
	% total lipids	13.5 (10.9, 15.2)	10.8 (9.2, 11.9)	12.7 (11.2, 15.1)	10.4 (8.9, 12.5)	9.3 (7.4, 11.6)	17.6 (16.0, 20.3)
Fibre	g/day	17.0 (14.2, 21.6)	10.5 (8.2, 14.6)	12.4 (10.1, 15.9)	10.9 (9.6, 15.0)	14.8 (9.8, 17.0)	14.0 (11.5, 16.6)
Ca	mg//day	1100 (889, 1401)	775 (615, 895)	895 (691, 1084)	752 (643, 999)	431 (232, 777)	855 (603, 979)
Fe	g/day	11.2 (9.9, 17.6)	9.1 (6.8, 11.7)	10.4 (7.7, 12.2)	7.5 (5.7, 10.5)	9.2 (6.8, 16.7)	8.7 (6.8, 12.0)
Na	mg/day	3420 (2865, 3924)	1362 (922, 2215)	2371 (1808, 2683)	1726 (1251, 2573)	1467 (808, 1848)	1712 (1384, 2032)

*Intake expressed as median g/kg/d and median % and the IQR of total energy intake. *CH*, carbohydrates; *SFA*, saturated fatty acids; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *Ca*, calcium; *Fe*, iron; *Na*, sodium.

3.2. Food groups' consumption frequency among centres

Table 4.9, shows the median frequency of food groups' consumption expressed in number of times consumed per day in the different centres. Milk and dairy products, were the most consumed group in all the centres (3 to 4 times/day), followed by starchy products (around 3 times/day). Meats were consumed 1-2 times/day, whilst fish and eggs were consumed less than once a day. In general, vegetables were consumed more than fruits in all the centres. The consumption of sweets and snacks was found to be 1-2 times/day. Oils were more consumed in Madrid, Valencia and Milan, while solid fats,

had an equivalent frequency of consumption in Leuven and Rotterdam, and Lisbon had a similar consumption of both types of fat. Legumes and nuts were the least consumed food groups in all the centres. Beverages showed a wide spectrum of consumption (0.4 to 3.4 times/day).

Table 4.9. Median daily frequency of consumption of food groups among children with Cystic Fibrosis in 6 European centres as assessed by means of a 4-day weighted food record. The 13 food groups were established by applying a common consensus criterion, which were assigned to the food items included in the national nutritional composition databases used.

Food Group (n times/day)	Lisbon	Madrid	Valencia	Milan	Leuven	Rotterdam	Median portion size per serving (g)
	n = 30	n = 33	n = 36	n = 30	n = 29	n = 49	
Milk & dairy	3.22	3.30	4.44	3.07	3.05	3.13	130.5
Meat products	1.63	2.13	2.76	1.39	2.03	1.44	77.1
Fish products	0.57	0.56	0.78	0.26	0.28	0.11	98.8
Egg products	0.21	0.35	0.42	0.11	0.23	0.06	50.4
Fruit products	1.19	1.03	1.10	1.47	0.99	1.35	83.3
Vegetable products	1.71	2.77	2.71	1.92	2.44	1.00	64.0
Legumes	0.18	0.19	0.19	0.11	0.01	0.02	70.6
Starchy products & grain	3.45	3.05	3.69	2.68	2.92	2.97	68.9
Beverages	0.81	0.42	0.61	0.72	2.01	3.40	118.2
Sweets & Snacks	1.19	0.99	1.89	2.40	2.11	2.04	52.1

Food Group (n times/day)	Lisbon	Madrid	Valencia	Milan	Leuven	Rotterdam	Median portion size per serving (g)
Oils	0.43	1.87	1.63	2.03	0.66	0.08	9.4
Solid fats	0.63	0.15	0.10	0.21	1.73	1.22	11.2
Nuts	0.02	0.04	0.23	0.00	0.16	0.21	42.8

3.3. Dietary origin of nutrients

3.3.1. Proteins

Meat products and milk and dairy were the major source of protein in all the centres, as shown in **Figure 4.8**. In Lisbon, Valencia, Leuven and Madrid meat was the first source, and in Milan meat and milk and dairy had a very similar contribution, whereas in Rotterdam milk and dairy and starchy products had a similar input than meat products. All the centres had in common the third source of protein, represented by the starchy products (except in Rotterdam where it was meat). However, two alternative patterns were described in the fourth group contributing to the total protein intake. In Lisbon, Valencia and Madrid, this was represented by the fish products, while sweets and snacks were the fourth source of protein in Leuven, Rotterdam and Milan. Eggs and nuts marginally contributed to the protein intake in all centres.

3.2.2. Carbohydrates

As expected, all centres had starchy products as the main source of carbohydrates (see **Figure 4.9**). Sweets and snacks were the second source of carbohydrates and milk and dairy the third in all the centres, except in Madrid where the tendency was the opposite. The contribution of fruit to carbohydrates intake was approximately twice the contribution of vegetables intake in all centres. Of note, legumes had a negligible representation. Sweeteners (sugar, honey and other sugar-based products) also provided a variable contribution in all the centres, being higher in

Valencia, Madrid, Rotterdam and Leuven than in the other centres. Focusing on the sugar-type carbohydrates, starchy products had a minor contribution to sugar, whilst milk and dairy, sweets & snacks, fruit and sweeteners were the main sources of sugar.

3.3.3. Lipids

The dietary sources of the lipid intake showed different patterns among centres (**Figure 4.10**). In Lisbon and Valencia, meat lipids contributed the most, and milk and dairy products were the second source of lipids. In the rest of the centres, milk & dairy was the first source of lipids, oils being the second source in Madrid and Milan, and sweets & snacks in Leuven and Rotterdam, and in all these centres meat was the third source of lipids, except in Milan. In Valencia, Madrid and Milan the lipids from oils contributed more than fats to the total lipid intake, whilst in Lisbon, Leuven and Rotterdam this tendency was found the opposite.

Regarding the type of lipids, the contribution of meat products to SFA and MUFA intake was in a similar proportion in all the centres, and this was also noted for the “sweets and snacks”. Milk and dairy was the group contributing the most to the SFA intake in all the centres. MUFA were provided mainly by oils in Madrid, Valencia and Milan, while the main sources of this type of fatty acid were “meats” and sweets and snacks in Lisbon, Leuven and Rotterdam. Finally, the highest PUFA intake was associated to meat, sweets and snacks and starchy products in all the centres but overall its intake was evenly distributed among all the food groups. Of note, nuts or fish did not contribute to the total lipid or PUFA intake.

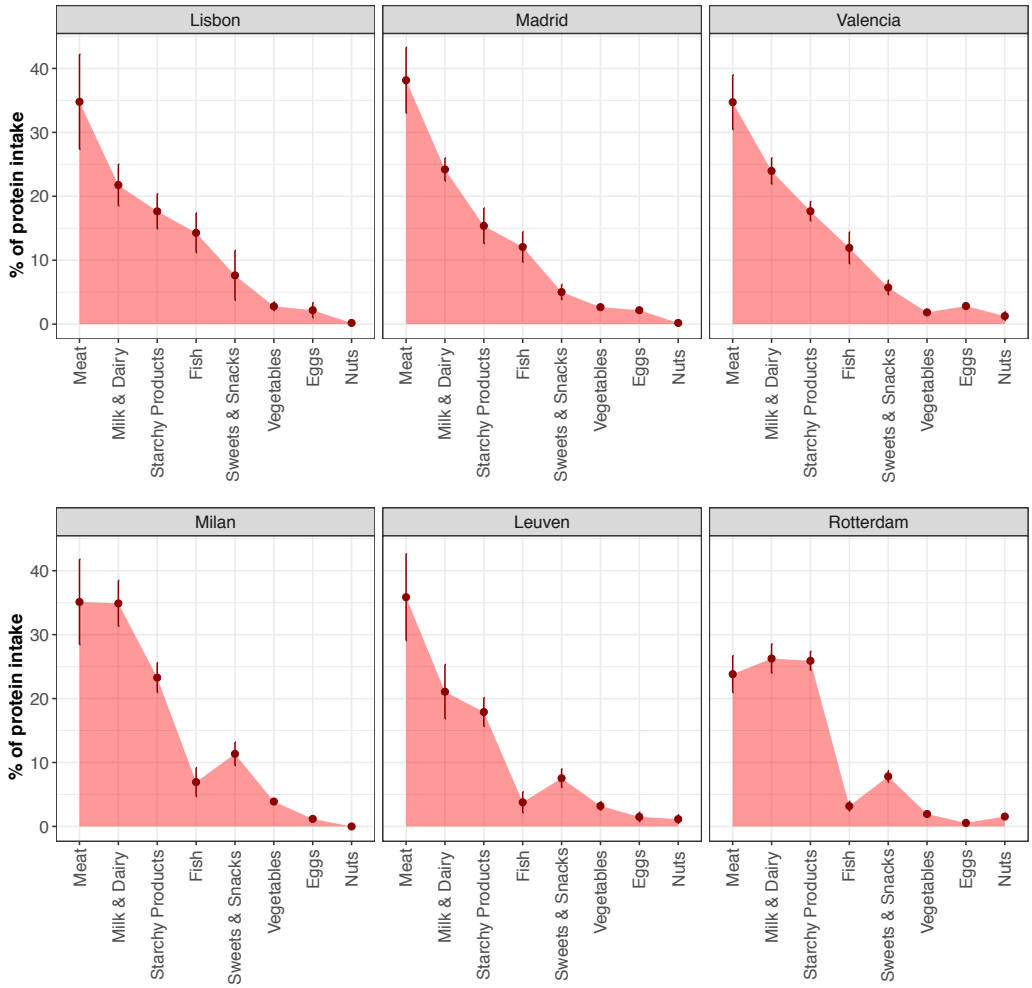


Figure 4.8. Contribution of food groups to daily total protein intake in a multicentre paediatric population of children with cystic fibrosis (n= 207). Dots represent the mean % of total protein coming from a food group/day and vertical bars represent the standard error.

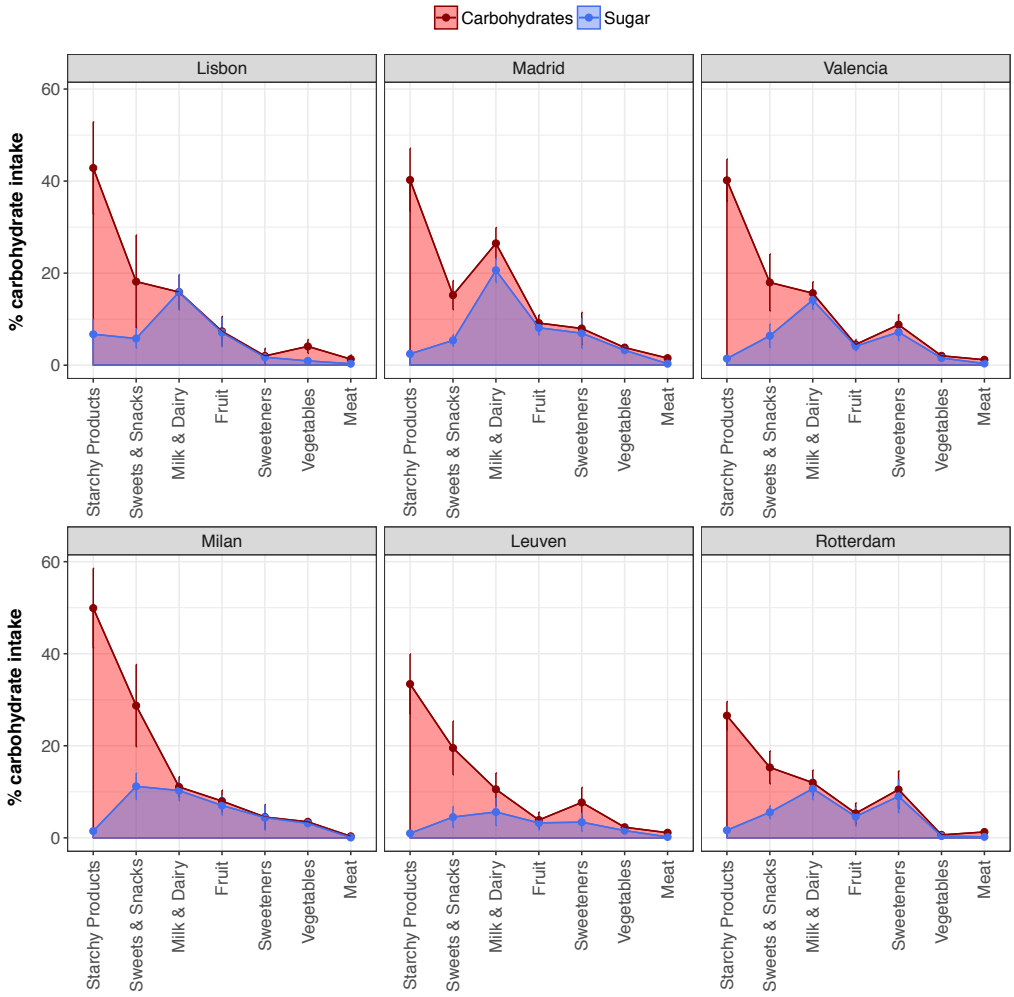


Figure 4.9. Contribution of food groups to daily total carbohydrates (red area) and sugar (blue area) intake in a multicentre paediatric population of children with cystic fibrosis (n= 207). Dots represent the mean % of total carbohydrates/sugars coming from a food group/day and vertical bars the standard error.

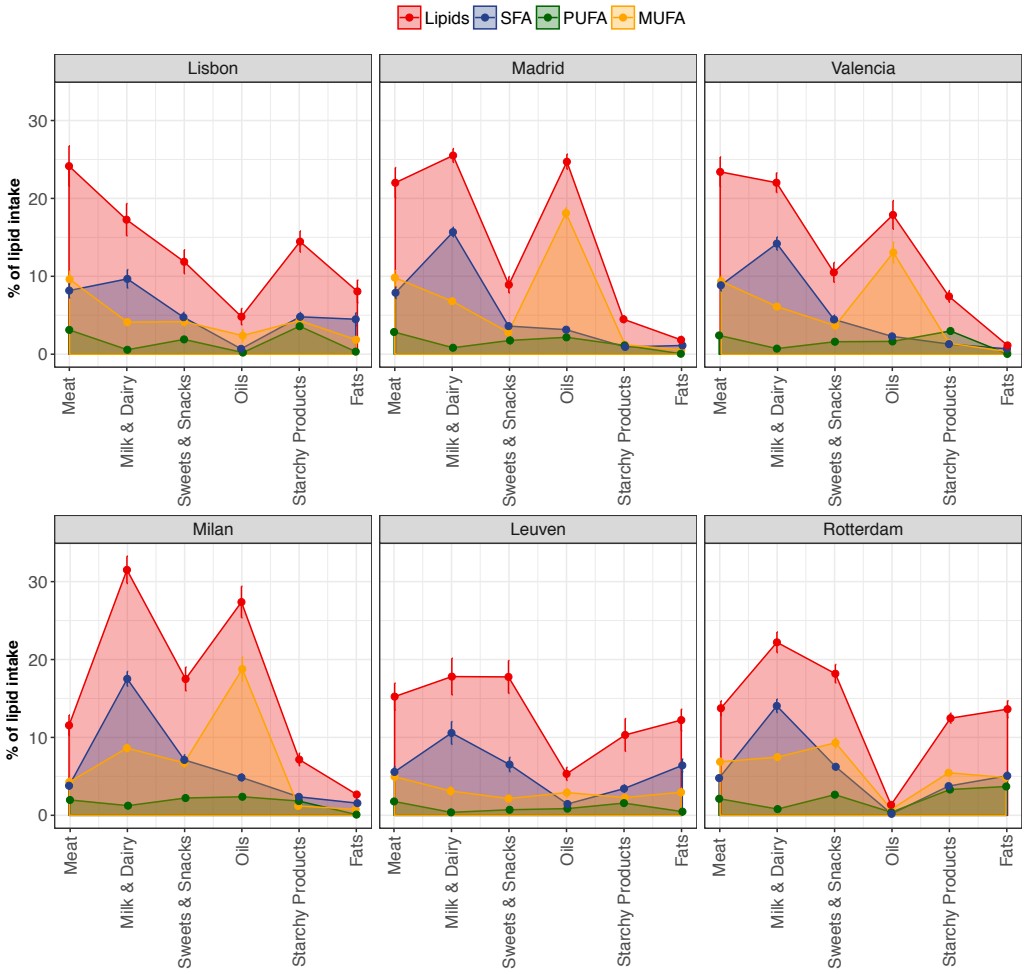


Figure 4.10. Contribution of food groups to daily total fat intake (red area), and to SFA (blue area), PUFA (green area) and MUFA (yellow area) intake in a multicentre paediatric population of children with cystic fibrosis (n= 207). Dots represent the mean % of total fats coming from a food group/day and vertical bars the standard error

4. DISCUSSION

The present European study in which a detailed assessment of consumption of food groups, macro and micronutrient intake, and dietary origin of the macronutrients in terms of food groups, was carried out in children with CF, and revealed important findings for future clinical practice. The main results concerning nutrient intake were a high intake of SFA with low PUFA and MUFA, and high sugar consumption in all centres. Moreover, considerable differences among centres were found in terms of sources of protein and lipid intake.

Our results also revealed a remarkably low consumption of fruit, vegetables, legumes, eggs and fish, whereas sweets and snacks were overconsumed. These results are in agreement with previous reports from studies in the general paediatric population over the last two decades; Brown et al. (1998) [11] concluded that processed products and fast-food style meals were prevalent in the general young population, and Braithwaite et al. (2017) [12] reached the same conclusion in their multi-country /centre study in children and adolescents.

The combined presentation of data as nutrients intake, as well as food groups' intake in our multicentre population, has enabled to identify and explain the base of the nutritional composition of the diets in children with CF enrolled in our study. For example, patients from Lisbon had the highest intake of protein related to the relatively high intake of meat and fish, in contrast to the patients from Rotterdam who had a low consumption of these food groups, thus reporting the lowest protein intake. Children from Milan presented a low intake of lipids due to the small intake of meat and processed products. On the other hand, children from Madrid and Valencia had a higher intake of lipids related to the high consumption of oils, and consequently they had the highest MUFA intakes. The Belgian patients also achieved a similar lipid intake, but mainly from processed products and solid fats. Low intake of PUFA was related to low intake of fish and nuts, the main source being meat and sweets and snacks.

The present study provides valuable indications for clinical practice, because the identification of the dietary origin of nutrients can set the basis for more targeted

dietary intervention, consisting in advising patients on how to achieve the recommended nutritional intake (lipids, protein, etc.), and at the same time reaching the national recommendations for a healthy diet.

The current described situation in children with CF should be considered a priority in developing educational material to guide patients' self-management and in promoting individualized dietary advice by health-care professionals. The most important general recommendations according to the results of this study are: 1) reducing SFA intake and increasing PUFA intake by means of a lower consumption of sweets and snacks, replacing them by nuts, and by increasing fish intake; 2) reducing sugar intake while maintaining or slightly decreasing the total carbohydrates intake by limiting the consumption of sweets and snacks and replacing these by fruit; 3) increasing protein intake by increasing the consumption of fish and nuts; 4) increasing fibre intake by means of promoting consumption of legumes, vegetables and nuts.

The main strengths of the present study rely on the robust methodology. First, participants have completed a specifically developed 4-day food record in which dietary intake was reported in a very detailed way. According to different bodies (e.g. EFCOSUM Project [13] and new European CF guideline [5]), the food record method is applicable in large European populations and can be considered as the best method to obtain population mean nutritional intakes and distributions [11]. The second and complementary strength is that a tailored nutritional composition database was used for the calculations, including food group categories, the whole range of macronutrients and a few micronutrients. Food groups and nutritional facts were harmonised and revised according to common criteria among the different countries, which overall allowed for a reliable comparison among populations. This is a crucial point, as so far methods to measure food intake were not standardised across Europe and intake data were generally poor, with uncertainties over the true nutrient intakes of children and adolescents [14]. Indeed, a recent study comparing the food intake among populations of two multicentre projects, encountered a major limitation for the comparison precisely because of the lack of a common criterion to classify food items

into groups [15]. The lack of adequate resources to conduct this type of studies could be the reason why scarce literature on food and nutrient intake is currently available.

Therefore, the methodological tools developed through the present study will allow, along the course of MyCyFAPP Project, for the accurate, long-term, continuous assessment and monitoring of the nutritional intake our CF population taking into account a wide range of nutrients. This unprecedented system of nutritional follow-up may be also applied in the future to other diseases at risk for nutritional imbalances, such as iron deficiency, obesity or diabetes [16,17], and even to the general population.

A limitation of our study relates to the lack of homogeneous criteria for recommended food groups' intake among the European countries, as previously acknowledged by the WHO (Food based dietary guidelines in the WHO European Region [18]). The nutritional intake recommendations are frequently based on different units, and expressed differently in various countries, i.e. the number of times or number of pieces [18]. Therefore, comparative analyses to a common standard or to the national recommendations cannot be performed adequately. Another limitation relates to the small amount of information found regarding micronutrient content in the product labels and in some of the nutritional composition databases used; therefore, in our NCDBs we often had to estimate/extrapolate the content from other similar products.

In conclusion, this study provides a full description of nutrients and food groups' intake in children and adolescents with CF from different European countries, based on specifically developed methodological tools. Imbalances in nutrient and food groups' intake were found in all centres, generating general and country-specific advices on how to improve nutritional adequacy.

ACKNOWLEDGEMENTS

The authors of this paper, on behalf of MyCyFAPP consortium, acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme for fully funding the project (ref. 643806).

Author contributions

JCL and CRK designed the study. JCL, TM, MR, EM, SW, IC, IA, MG, AB, SW, PC and LV collected the data. VFF performed the statistical analysis. JC, JH and MB wrote the first draft with contributions from CRK. All the authors reviewed and commented on subsequent drafts of the manuscript. All the authors approved the submitted version.

Funding

Project fully funded by the European Union - Horizon 2020 Research and Innovation Framework Programme (ref. 643806).

Conflict of interest

No conflicts of interest to be declared.

5. REFERENCES

1. Baker, R. D. (2013) Assessing Exocrine Pancreatic Function : When the Best Test Is Not Possible. J Pediatr Gastr and Nutr 56:116–117.
2. Rovner, A. J., Schall, J. I., Mondick, J. T., Zhuang, H. & Mascarenhas, M. R. (2014) Delayed small bowel transit in children with cystic fibrosis and pancreatic insufficiency: Commentary. Inflamm. Bowel Dis. Monit. 14:65–66.
3. Woestenenk, J. W., Ent, C. K. Van Der & Houwen, R. H. J. (2015) Pancreatic Enzyme Replacement Therapy and Coefficient Cystic Fibrosis. J. Pediatr. Gastroenterol. Nutr. 61:355–360.
4. Borowitz, D. et al. (2007) Coefficients of Fat and Nitrogen Absorption in Healthy Subjects and Individuals with Cystic Fibrosis. J Pediatr Pharmacol Ther 12:4–9.
5. Turck, D. et al. (2016) ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. Clin. Nutr. 35:557-577
6. Panagopoulou, P., Fotoulaki, M., Nikolaou, A. & Nousia-arvanitakis, S. (2014) Prevalence of malnutrition and obesity among cystic fibrosis patients. Pediatr. Int. 56:89–94.

7. Hanna, R. M. & Weiner, D. J. (2015) Overweight and obesity in patients with cystic fibrosis: A center-based analysis. Pediatr. Pulmonol. 50:35–41.
8. Sutherland, R. E., Collins, C., Brunner, R., Ooi, C. Y. & Katz, T. E. (2017) 316 An historical perspective of dietary intake studies in children with CF. J. Cyst. Fibros. 16:S143.
9. Calvo-lerma, J. et al. (2017) Innovative approach for self-management and social welfare of children with cystic fi brosis in Europe : development, validation and implementation of an mHealth tool (MyCyFAPP). Br. Med. J. Open 7:e014931
10. Calvo-Lerma, J. et al. (2017) Nutritional status, nutrient intake and use of enzymatic supplements in paediatric patients with Cystic Fibrosis; a multicentre study with reference to current guidelines. J. Cyst. Fibros. 16:510-518
11. Brown, K., Mcilveen, H. & Strugnell, C. (2000) Nutritional awareness and food preferences of young consumers. Nutrition & Food Science, 30:230-235.
12. Braithwaite, I. et al. (2017) Body Mass Index and vigorous physical activity in children and adolescents: an international cross-sectional study. 0–3 Acta Paediatrica, doi:10.1111/ijlh.12426
13. Biró, G., Hulshof, K., Ovesen, L. & Amorim, J. A. (2002) Selection of methodology to assess food intake. Eur. J. Clin. Nutr. 56:S25
14. Lambert, J. et al. (2004) Dietary intake and nutritional status of children and adolescents in Europe. British Journal of Nutrition, 92:S147-S211
15. Stefler, D. et al. (2015) Comparison of food and nutrient intakes between cohorts of the HAPIEE and Whitehall II studies. The European Journal of Public Health, 26:628-634
16. GB, M., RA, H. & LA., L. (2013) Canadian Diabetes Association: Clinical Practice Guidelines Expert Committee, Dyslipidemia. Can. J. Diabetes 37:S110–16
17. Mesías, M., Seiquer, I. & Navarro, M. (2013) Iron nutrition in adolescence. Crit. Rev. Food Sci. Nutr. 53:1226–337.
18. Food based dietary guidelines in the WHO European Region. (2004).

CHAPTER 3

EFFECT OF FOOD INTRINSIC AND EXTRINSIC FACTORS ON LIPID DIGESTION

PAPER 5

Calvo-lerma, J. et al. (2018) The role of gastrointestinal conditions on *in vitro* lipids' digestion. Clinical Nutrition, Under review

PAPER 6

Calvo-lerma, J. et al. (2018) *In vitro* digestion of lipids in real foods: influence of lipid structure within the food matrix and interactions with non-lipid components. Journal of Food Science, Under review

PAPER 7

Calvo-lerma, J. et al. (2018) *Lipolysis of oil and butter under joint in vitro digestion of carbohydrate and protein rich food matrices.* Food & Function, Under preparation

PAPER 5

THE ROLE OF GASTROINTESTINAL CONDITIONS ON IN VITRO LIPIDS' DIGESTION

Joaquim Calvo-Lerma[a,b*], Victoria Fornés-Ferrer[b], Irene Peinado[a], Carmen Ribes-Koninckx[b,c], Ana Heredia[a] and Ana Andrés[a]

[a] Universitat Politècnica de València. Instituto de Ingeniería de Alimentos para el Desarrollo. Camino de Vera s/n. 46022 Valencia (Spain)

[b] Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell 106. 46026 Valencia (Spain). Tel. +34 961246712

[c] Hospital Universitari i Politècnic La Fe de València. Servicio de Gastroenterología y Hepatología Pediátrica. Avenida Fernando Abril Martorell 106. 46026 Valencia (Spain). Tel. +34 961246712

ABSTRACT

Background & aims: Pancreatic Enzyme Replacement Therapy (PERT) palliates pancreatic insufficiency but shows different responses in patients with cystic fibrosis. The present study aims at assessing the influence of gastrointestinal conditions on lipolysis by means of an *in vitro* digestion model.

Methods: gastrointestinal digestion of a nutritional supplement was simulated. Different conditions of gastric and intestinal pH, bile salts composition and concentration, fat content in the digestion medium and the volumetric proportion of food/digestive fluids were assessed. PERT dose was set at 1000 Lipase Units/ g fat. The progress of intestinal lipolysis was measured with the pH-stat method. Lipolysis extent was expressed as % of free fatty acids released from the initial fat content. Lipolysis kinetics was modelled by log-logistic dose-response models.

Results: intestinal pH 7 was the condition with the highest effect on lipolysis followed by high amount of fat in the digestion medium. The composition of bile formula was also significant, the bovine and the high-taurocholic salts leading to the lowest lipolysis. At 1mM concentration of bile salts, lipolysis was significantly lower than at the 10mM. Finally, the proportion between food/ simulated digestive fluids had also an impact.

The model describing lipolysis kinetics (saturation rate and digestion asymptote) supported the results in terms of lipolysis extent.

Conclusions: gastrointestinal factors strongly determine PERT efficacy. Our findings could set the basis for establishing complementarily therapies to PERT and strongly encourage applying the existing well-defined adjuvant approaches in clinical practice.

KEYWORDS: cystic fibrosis, pancreatic enzyme replacement therapy, gastrointestinal conditions, *in vitro* digestion, lipolysis, bile salts

ABREVIATIONS

CI: confidence interval; CF: cystic fibrosis; g: grams; GC: glycocholic; GCDC: glycochenodeoxicholic; HFF: high fat food; HCl: chlorhydric acid; L: litre; LFF: low fat food; LU: lipase units; mL: mililitre; mM: milimolar; mmol: milimol; N: normality; NaOH: sodium hydroxide; PERT: pancreatic enzyme replacement therapy; PPI: proton pump inhibitor; SGF: simulated gastric fluid; SIF: simulated intestinal fluid; SSF: simulated salivary fluid; TC: taurocholic; TCDC: taurochenodeoxicholic; V: volume; V/V: volume/volume

1. INTRODUCTION

Pancreatic insufficiency (PI) is an associated disorder secondary to Cystic Fibrosis (CF) affecting 85-90 % of the patients [1]. The obstruction of the pancreatic duct impairs pancreatic enzymes release in the duodenum, what specially compromises lipolysis [2]. To palliate the insufficiency, Pancreatic Enzyme Replacement Therapy (PERT), consisting on the exogenous administration of a porcine-origin enzyme supplement, is used to promote nutrients' digestion and absorption and avoid malnutrition [3]. The new European Guidelines in CF acknowledge low evidence in the recommendation for PERT dose [4], with a wide background of studies that unsuccessfully could explain the lack of association between dose and body mass index

or coefficient of fat absorption [5,6]. Besides, a recent study revealed that, despite very different dosing criterions were being applied among European countries, there were no differences in the nutritional status of CF children [7]. In addition, this study evidenced the wide intra-patient variability in the dose for different meals, suggesting that different doses could be the optimal ones according to patients' individual conditions [7].

Nutritional status has proven to be the most associated factor on CF disease prognosis and survival, and growth stunting can only be avoided by accurate PERT dosing [8,9]. Therefore, special interest should be given to unveil mechanisms underpinning PERT activity during digestion in order to address effective clinical interventions.

There are studies pointing the food characteristics as important factors affecting the role of pancreatic enzymes activity [10,11,13], while some others place the focus on the gastrointestinal conditions as the main determinants [13–17]. In the particular case of CF, specific differences as compared to the healthy population take place in the intestine (pH and bile concentration) due to the pancreatic insufficiency and altered biliary function [15,17,18,19], leading to a wide range of possible sets of individual gastrointestinal conditions. However, measuring these parameters in patients implies highly invasive techniques plus considerable ethical restrictions.

In this context, *in vitro* digestion models might suppose a useful tool for simulating different gastrointestinal environments in lab, under controlled, accurate and reproducible conditions [11,12]. Thus, studies in this setting would allow for examination of the role of different factors on lipid digestion, and thus provide explanations on different lipolysis responses and eventually different PERT requirements [20–22].

In the present scenario, MyCyFAPP Project (www.mycyfapp.eu) pursues CF patients' self-management of PERT dosing supported by a mobile application [23]. Within the frame of MyCyFAPP, the work presented is aimed at elucidating the role of gastrointestinal conditions on lipolysis by means of an *in vitro* digestion model.

2. MATERIALS AND METHODS

2.1. Materials and equipment

A test food, i.e. a nutritional supplement, was used to conduct the experiments (Resource CF®). The nutritional information of the product included: protein content solely from casein, lipid content from medium-chain triglycerides and no phospholipids. Pancreatic enzyme supplements (ES) (Kreon® 10,000 LU) were obtained from Hospital Universitari i Politècnic La Fe (Valencia, Spain). Pepsin from porcine gastric mucosa (3200-4500 U/mg), bovine bile extract, porcine bile extract, taurocholic (TC), taurochenodeoxycholic (TCDC), glycocholic (GC) and glycodeoxycholic (GCDC) compounds were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Chlorhydric acid 1N and sodium hydroxide 1N were used to adjust the pH at the different digestion stages.

The pH-stat method was applied to conduct all the experimental trials. A 718 STAT Titrino (Methrom) connected to the software Tiamo 1.3 was used. This equipment allows for automating acid-base reactions. The sample is introduced in the reaction vessel connected to a thermostated water bath. In the vessel, pH and temperature electrodes are placed, along with an automatic dosing tube pouring the titrant. Lipolysis during the intestinal stage was measured with the "stat pH" function, in which the equipment adds titrant automatically when a pH change is produced in the reaction vessel - due to the lipolysis reaction - in order to maintain the constant desired pH in the medium. As the digestion process occurs, the equipment registers every 10 seconds the volume of titrant consumed over time. Then, the added volume at any point of the process can be translated into the amount of free fatty acids released as the product of lipolysis.

In order to discard possible titration effects derived from proteolysis during the intestinal stage, a complementary experiment was conducted without enzymatic supplement and with pancreatic proteases and no pH changes were detected. Thus, in our setting, changes in pH along the intestinal stage can be attributed to the sole effect

of lipolysis since complete proteolysis of the casein in the test food occurs during gastric stage by the action of pepsin [24].

2.2. Study design

Three sets of experiments were carried out in order to elucidate the role of the extrinsic factors: gastric pH, intestinal pH, bile concentration, bile salts composition, volume of digestive fluids and fat in the digestive medium. The combinations of the study variables for the experiments that were tested are shown in **Table 4.10**. They were selected according to the altered parameters described in CF [17,25–27]. For all the experiments, the amount of digestive enzymes was 1000 LU/g of fat, based on preliminary kinetics study [19] that revealed that it was the optimal dose for the conditions intestinal pH 7 and bile salts bovine 10mM. All the experiments were done in triplicate.

a) Gastric and intestinal pH: the gastric stage was conducted at pH 3, 4 and 5 in order to simulate the effect of proton pump inhibitors (PPI). Then the intestinal stage was simulated both at pH 6 with bile salts 1mM concentration (CF affected scenario) and at pH 7 with bile salts 10 mM (normal conditions).

b) Formulation and concentration of bile salts: four formulations were prepared (**Annex 2**): bovine bile (formula 1, F1), porcine bile (formula 2, F2), low-taurocholic and high-glycocholic salts bile (formula 3, F3) and high-taurocholic low-glycocholic salts bile (formula 4, F4).

c) Food/fluids dilution factor: three different proportions of digestive fluids / food sample were tested: 1/1, 0.5/1 and 2/1 in the three digestion stages, in order to experiment different concentrations of enzymes in the volume. In addition, two formulations of the food sample were tested with 5.5 % (low fat food, LFF) and 35% of fat (high fat food, HFF), by mixing different amount of the food powder with water, in each of the digestive fluids proportion in order to assess different concentrations of substrate (lipids) in the volume.

Table 4.10. Experimental design: combination of the gastrointestinal conditions as study variables and resulting fat and enzyme concentrations in the digestion medium

Gastric pH	Intestinal pH	Bile formula	Bile concentration (mM)	Ratio Food sample/ digestive fluid (v/v)	Fat (g/mL)	Enzyme (LU/mL)	Food fat composition (%)
3	6	F1	1	1/1	0,0079	7.91	5.5
3	7	F1	10	1/1	0,0079	7.91	5.5
4	6	F1	1	1/1	0,0079	7.91	5.5
4	7	F1	10	1/1	0,0079	7.91	5.5
5	6	F1	1	1/1	0,0079	7.91	5.5
5	7	F1	10	1/1	0,0079	7.91	5.5
3	6	F2	1	1/1	0,0079	7.91	5.5
3	6	F2	10	1/1	0,0079	7.91	5.5
3	6	F3	1	1/1	0,0079	7.91	5.5
3	6	F4	1	1/1	0,0079	7.91	5.5
3	6	F1	1	0.5/1	0.0159	15.9	5.5
3	6	F1	1	2/1	0.0039	3.9	5.5
3	6	F1	1	0.5/1	0.1	100	35.0
3	6	F1	1	1/1	0.05	50	35.0
3	6	F1	1	2/1	0.025	25	35.0

mM, mili molar; F1, formula 1, bovine bile; F2, formula 2, porcine bile; F3, formula 3, high in glycocholic salts bile; F4, formula 4, high in taurocholic salts bile; mL, millilitre; LU, lipase units.

2.3. *In vitro* digestion process

The digestion process was simulated according to the static standardised method proposed by Minekus et al., (2014) [19] which establishes the “smallest common denominator” of the standard conditions that are close to the physiology of a healthy adult, and thereafter amendments were applied according to the scope of this research [12]. The static digestion process was simulated in three stages.

Oral stage: The food sample (5 ml) was mixed in the study proportion (v/v) with simulated salivary fluid (SSF) in the digestion vessel during 2 minutes at 37 ºC.

Gastric stage: Then, simulated gastric fluid (SGF) (pH 3) was added in the study proportion (v/v) to the digestion vessel containing the oral bolus. The pH of the mixture was readjusted according to the experimental set with HCl (1N). Pepsin solution was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). At this point, the ES was added in a concentration of 1000 LU/g fat. The sample was stirred at 55 rpm for 2 h at 37 ºC, simulating the physiological process. Of note gastric lipase was not added because it is not commercially available, only fungal lipases can be obtained which exhibit different activity and specificity. Moreover, in physiological conditions lipase activity is much lower in the gastric than in the intestinal stage, because optimal gastric lipase pH is around 5; thus its contribution to total lipolysis can be neglected [12].

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF) (pH 6, was added to the vessel containing the gastric chyme in the proportion (v/v) according to the experimental design. Bile salts solution was added to the SIF in order to reach the desired final concentration in the intestinal mix depending on the experimental design. The pH of the mixtures was adjusted with NaOH (1N). The samples were then stirred at 55 rpm for other 2 h at 37 ºC. pH was maintained during the process by the automatic addition of NaOH 0.5N.

Fluids' composition required for each digestion stage, were those described by Minekus et al., 2014 (1) (**Annex 3**); they were prepared fresh daily and kept at 37 ºC before their use.

2.4. Lipolysis extent calculation

The percentage of free fatty acids released as referred to the initial amount of lipids of the sample was used to express the extent of lipolysis; it was calculated on the basis of the NaOH consumed during the intestinal stage (during pH-stat) as referred to

the molecular weight of oleic acid, for this being the predominant fatty acid in the formulation of the food sample (**Equation 1**).

The curves of the progress of lipolysis for all the assessed conditions in the study were obtained by calculating the % of lipolysis every 10 seconds along 120 minutes.

$$\%\ lipolysis = \frac{(V\ NaOH)(N\ NaOH)(MW\ oleic\ acid)(100)}{m\ substrate} \qquad (\textbf{Equation 1})$$

Where: V NaOH = titrant volume at any point (L); N NaOH = concentration of the titrant (N); MW oleic acid = molecular weight of the oleic acid; m substrate = mass of fat in the food sample (g).

2.5. Statistical analysis

For the descriptive analysis, the data were summarised using mean (standard deviation) or median (1^{st}, 3^{rd} Q.) in the case of continuous variables and with relative and absolute frequencies in the case of categorical variables.

Beta regression models were applied in order to explain the association of the study variables (gastric pH, intestinal pH, bile concentration, bile formulation, volume of digestive fluids, fat concentration) with the response variable, i.e. the lipolysis extension (%). The results of the model can be interpreted with the exp(estimate) and the CI 95%. If the exp(estimate) is >1 means that the variable is positively associated with the response variable, i.e. lipolysis extent, and if <1 the effect is diminishing of the response variable. The higher the value, the higher the effect. Complementarily, the confidence intervals that do not contain 1 are those significantly associated with the response variable.

To analyse the kinetics of lipolysis, log-logistic dose-response models were adjusted to estimate the parameters that describe the time-effect on the lipolysis extent (f(x)) as an asymptotic curve. Several models were fitted for each condition in

each experiment and each of them provided the three-parameter log-logistic function (**Equation 2**) where the lower limit is equal to 0. The numerator "d" refers to the estimated asymptote (i.e. lipolysis extent) while the parameter "e" represents the saturation rate. Finally, “b” represents the activation time. The higher the value, the lowest the saturation speed during the *in vitro* digestion process, conversely, the lowest the value, the higher the saturation speed/fat absorption.

$$f(x) = \frac{d}{1 + \exp\left(b(\log(x) - \log(e))\right)} \qquad \textbf{(Equation 2)}$$

Where: d = estimated lipolysis extent asymptote; b = activation time; e = activity saturation rate

All the analyses were performed using R software (version 3.3.3), and betareg (version 3.1-0), drc (version 3.0-1) packages. A p-value below 0.05 was considered statistically significant.

3. RESULTS

3.1. Effect of gastric pH and intestinal pH on lipolysis extent

As shown in **Figure 4.11a**, gastric pH 4 was associated with the highest lipolysis extent at the intestinal stage as compared to the other gastric conditions (p 0.027, CI95% [1.04, 2.10] (**table 4.11**), the impact of a gastric pH 5 not being significant (p 0.08). It was the intestinal pH, however, the variable causing the highest effect on lipolysis extent (CI 95% [16.35, 31.94]), significant differences existing between intestinal pH 6 and 7 ($p<0.001$).

3.2. Effect of bile composition and concentration on lipolysis extent

As shown in **Figure 4.11b**, at 1mM concentration, with the porcine (F2) and low-taurocholic (F3) bile formulas, lipolysis reached values of 39 and 40% respectively, while the bovine (F1), and the high-taurocholic (F4) allowed for mean values of 32 % and 30

% respectively. When analysing differences (**Table 4.10b**), compared to F1, F4 showed a decreasing effect (0.88, [0.76, 1.02]) but it was not significant, and the positive effect on lipolysis of F2 and F3 was significantly higher (p 0.017 and p<0.001 respectively).

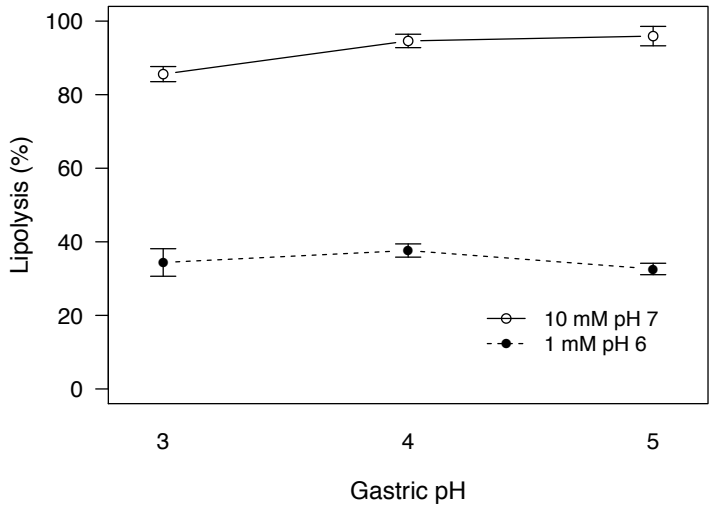
The concentration of bile, 1 mM vs 10 mM, was also studied for F1 and F2. This study (**Table 4.10c**) showed that bile concentration 10 mM favoured lipolysis significantly in both compositions (CI 95% [1.37, 1.78], p<0.001). Furthermore there was a significant interaction between the composition and the concentration of the bile (p=0.017), provided that the effect of the concentration 10 mM was higher in bovine bile (F1), than in porcine bile (F2) (CI 95% [0.66, 0.96]).

3.3. Effect of the fat concentration in the digestive medium

The influence of different fat concentration in the simulated digestive medium was assessed, by comparing the LFF *vs.* the HFF. As the enzyme amount was established in terms of LU per gram of fat, depending on the fat content of the sample food, different enzymes concentration resulted as well. In addition, different proportions of simulated digestive fluids/food sample were assessed, resulting in six different combinations of concentrations of fat and enzymes in the digestion medium. As shown in **Figure 4.11c**, the HFF had the highest positive effect on lipolysis as compared to the LFF (CI 95% [5.62, 8.13] p<0.001]. The proportions 0.5/1 and 2/1 were not associated with a significant change in lipolysis in the case of LFF (**Table 2d**), but there was a significant interaction in the case of HFF with these proportions, in which lipolysis was lower in the proportions 0.5/1 and 2/1 as compared to the 1/1 proportion (p <0.001 and p 0.002 respectively).

Table 4.11. Linear mixed regression models to assess the effect of the gastrointestinal conditions on lipolysis.

Variable	Estimate	95% Confidence interval		p-value
Gastric pH4	1.482	[1.045	2.101]	0.027
Gastric pH5	1.366	[0.964	1.937]	0.08
Intestinal pH7	22.858	[16.357	31.942]	<0.001
R-squared 0.934				
Bile formula 2	1.19	[1.03,	1.38]	0.017
Bile formula 3	1.30	[1.12,	1.50]	<0.001
Bile formula 4	0.88	[0.76,	1.02]	0.086
R-squared 0.737				
Bile formula 2	1.19	[1.04,	1.36]	0.01
Bile10 mM	1.56	[1.37,	1.78]	<0.001
Interaction bile formula 2 : bile 10 mM	0.79	[0.66,	0.96]	0.017
R-squared 0.823				
HFF	6.764	[5.626	8.132]	<0.001
0.5/1 (v/v)	0.914	[0.769	1.087]	0.309
2/1 (v/v)	0.966	[0.813	1.147]	0.69
Interaction HFF : 0.5/1	0.568	[0.442	0.731]	<0.001
Interaction HFF : 2/1	0.665	[0.516	0.857]	0.002
R-squared 0.982				



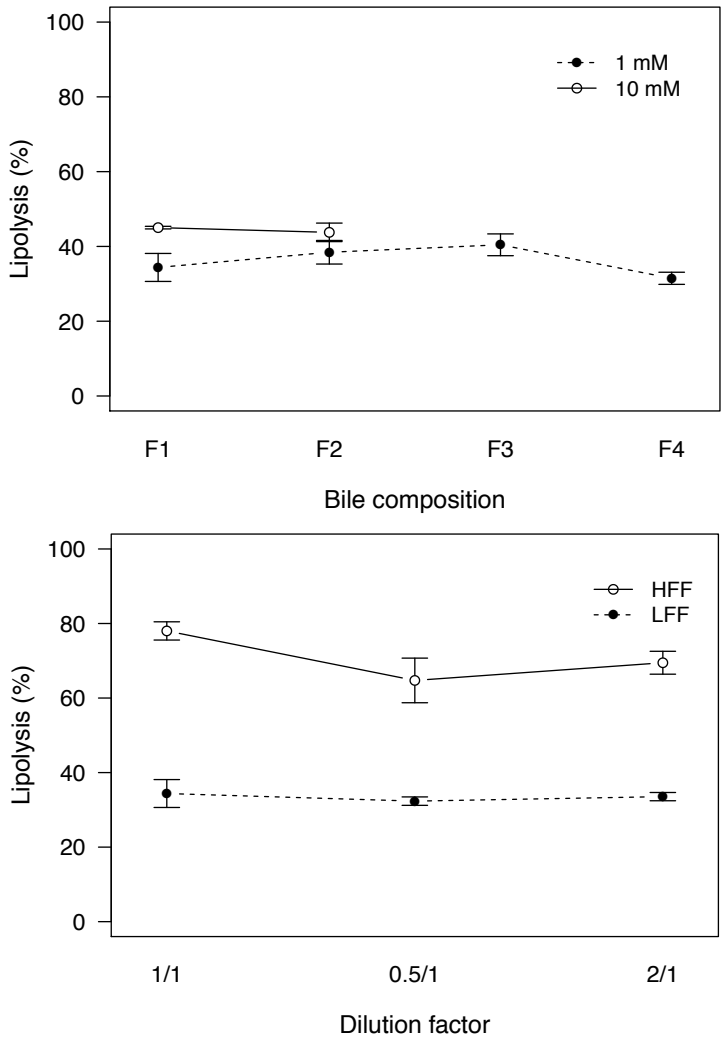


Figure 4.11. Lipolysis extent at the end of intestinal digestion (mean and standard deviation) **1a)** after gastric digestion at three different pH (3, 4 and 5) and in two sets of intestinal conditions: healthy (pH7 and 10mM bile salts) and CF-affected (pH6 and 1mM bile salts); **1b)** using four bile compositions (F1, bovine bile salts; F2, porcine bile salts; F3, high-glycocholic bile salts; F4, high-taurocholic bile salts) and in two concentrations of bile salts (1mM and 10mM) for F1 and F2; **1c)** using three different ratios of food/simulated digestive fluids, and two concentrations of fat in the digestion medium (HFF, high fat food and LFF, low fat food)

3.4. Lipolysis kinetics assessment

A complementary analysis was applied using the data from the lipolysis process in order to assess kinetics. The defining parameters of these time-response curves were obtained and thus, the effect of the experimental conditions could be attributed to the process of lipolysis. **Annex 4** shows the results.

Figure 4.12a shows that in normal conditions (intestinal pH 7, bile salts 10mM) lipolysis extent was higher than in affected conditions (pH 6, 1mM). Given that the asymptote of the function in normal conditions reached values close to 90-100% (CI95% [91.6, 92.6], [98.4, 99.6] and [101.5, 102.8]) and there was no overlapping with the confidence intervals in affected conditions (CI95% [41.6, 44.9], [47.9, 51.5] and [37.8, 39.4]), for gastric pH 3, 4 and 5 respectively, a significantly higher lipolysis in normal than in affected conditions was confirmed, reaffirming the results shown in section 3.1. Likewise, the parameter "e" values (saturation rate) and its confidence intervals were much lower (10 *vs.* 30) in normal conditions than in the affected, what means that the saturation rate is higher (as explained in section 2.6).

In **Figure 4.12b**, F1 and F2 reach a higher lipolysis extent (asymptote) in bile salts concentration 10mM than in 1mM (CI95% [52.5, 54.9], [63.3, 68.6] *vs.* [41.6, 44.9], [40.9, 42.4] respectively) as reported in section 3.2. Besides, at concentration 1mM, slight differences are observed between F2 and F3, which reached higher lipolysis than F1 and F4.

Finally, the third experimental set (**Figure 4.12c**) proved that HFF had a higher saturation rate "e" (i.e. in less time the asymptote was reached) than the LFF (CI 95% [31.1, 33.6], [27.4, 33.0] and [53.6, 66.9] for the food/ digestive fluids proportions 0.5/1, 1/1 y 2/1), and also higher lipolysis extent values (79.8, 125.2 and 114.1 in HFF *vs.* 39.2, 43.2 y 47.8 in LFF for the volumetric proportions 0.5/1, 1/1 and 2/1).

These complementary analyses were carried out to assess the sensitivity and to confirm the results obtained and reported in the sections above.

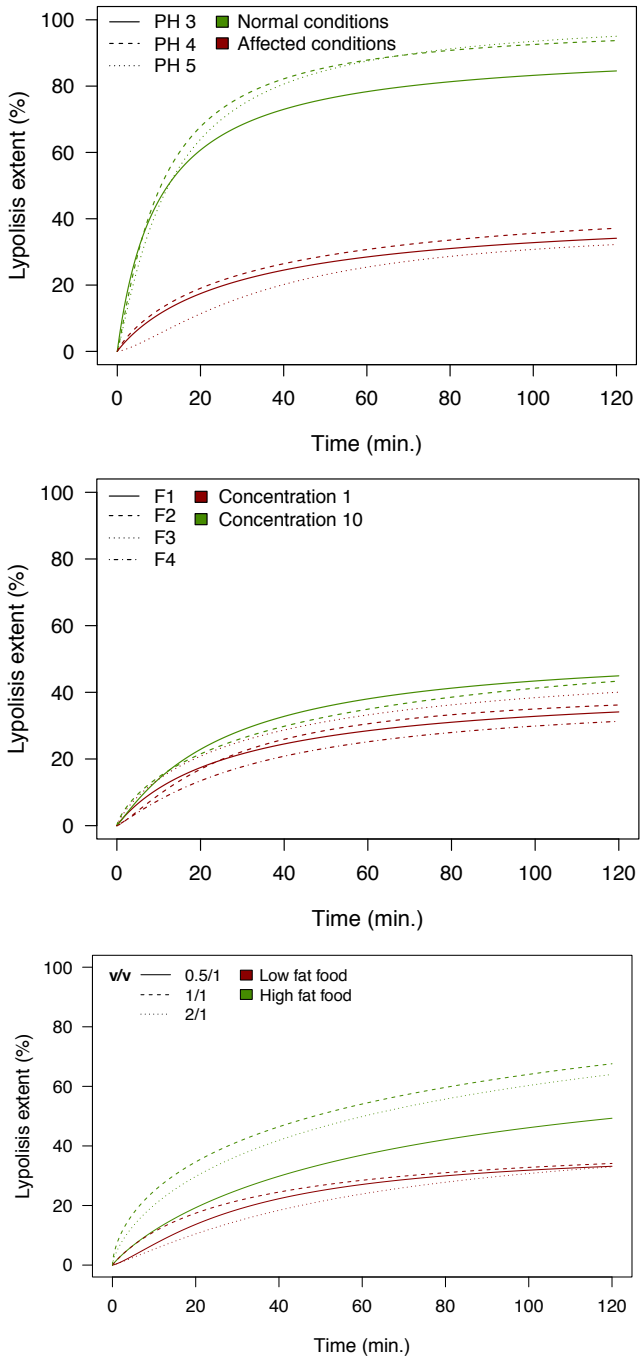


Figure 4.12. Lipolysis curves obtained with the predictive equations of the model. **a)** effect of gastric pH, and the combination of intestinal pH and bile salts concentration; **b)** effect of the bile composition and concentration; **c)** effect of the ratio of digestive fluid/food and the fat concentration of the food sample.

4. DISCUSSION

Through the present study we assessed the influence of several gastrointestinal conditions on lipolysis extent and kinetics by means of an *in vitro* digestion methodology. The intestinal pH was the condition showing the greatest incremental effect in lipolysis extent by far when comparing 6 *vs.* 7. The other conditions showing improved lipolysis were the high concentration of fat in the digestion medium, the bile salts concentration 10mM, the bile formulation with high glycocholic salts, and the proportion of food/ digestive fluids 1/1. Complementarily, the study of lipolysis kinetics reinforced the effects described by the statistical models developed on the basis of final lipolysis: the highest asymptotes were found in the conditions intestinal pH 7, bile 10 mM and high fat food, along with shortest time before reaching saturation.

The selection of the gastrointestinal conditions to be studied were based on a thorough literature search focused on studies in CF. In the stomach, pH is maintained at an average 3 during the gastric digestion [25,28], but we also simulated gastric stage at pH 4 and 5 to mimic the effect of proton pump inhibitors (PPI), which maintain higher values during this phase [29]. Intestinal pH values of 6 and 7 were selected on the basis of studies showing that the range in CF is between 4.5 and 6.5 [18,30–34]. We did not conduct our experiments at pH 4.5 – 5.5 since in preliminary assays from our group no enzymatic activity was detected. To determine the concentration and composition of bile salts to be simulated, we found controversial information in the literature. According to Minekus et al. 2014 the formulation of bile salts in an *in vitro* setting should allow for a concentration of 10mM in the intestinal digestion medium [12], but to address possible changes in the case of CF, only one study conducted with a robust methodology was found, which approached the topic integrally [26]: it proved that bile salts concentration could be up to 10 times lower in the case of CF and thus the 1mM condition was considered in our study. Results of this study also guided the formulation of the bile F3: high glycocholic salts bile described in CF; and F4: high taurocholic salts, would simulate a supplementation with taurine. Finally, the proportion of food/

digestive fluids in the medium was made based on the healthy physiological 1/1 proportion with foods [12], and half of this proportion was simulated given that pancreatic secretion in CF is reduced (up to half) as compared to healthy individuals [27] and the proportion 2/1 was used to see possible dilution effects.

Our results could partially explain why in the clinical practice, differences in the coefficient of fat absorption among patients with CF with similar characteristics have been found [35], for example individuals able to reach intestinal pH 7 would have low enzyme dose requirements to achieve optimal lipolysis. For practical and ethical reasons, assessing these differences in patients is difficult due to the highly invasive techniques required (gastric and duodenal aspiration at different digestion times), although there are some studies in the field [36]. Thus, we consider our *in vitro* experiment is a valid approach to reveal the role of the different gastrointestinal conditions - and their combinations - on lipolysis.

According to our findings, the intestinal pH is the most determining condition of lipolysis both in terms of lipids digestion rate before saturation and extent, as increasing pH from 6 to 7 led to an improvement of 54 % in lipolysis in the test food. A study with patients nasoduodenal intubated showed that intestinal pH was unequivocally associated with % of lipids digestion [30], what supports our results.

Thus, increasing intestinal pH in CF should be considered a therapeutic priority. In the clinical practice, the application of new CFTR regulatory therapies, such as Ivacaftor, showed an increased intestinal pH in patients but not as high as 7 [34]. PPI supplementation can also achieve it: their inhibitory effect on acid secretion in the stomach lead to a less acid gastric content so when the chyme passes into the small intestine, despite the low bicarbonate secretion, the pH increase at this stage is higher than when starting from a gastric chyme around pH 3. However, to our knowledge it is not sufficiently effective to reach a pH 7 along the entire intestinal stage. Despite this therapy has proven to improve digestion and absorption of nutrients and nutritional status in patients with CF, it is not common clinical practice [37-39]. A third approach to increase intestinal pH is related to the supplementation with sodium bicarbonate,

although results up to date have showed a neutral effect, possibly due to the coating system employed in the encapsulation of the compound [40].

The second factor with a relevant effect on lipolysis was the amount of fat in food, what is translated in fat concentration in the digestion medium. From a practical point of view, adherence to a high-fat diet is the current advice [4], although the motivation for this recommendation is the need to achieve a high caloric diet [4]. Our findings suggest that this improvement starts at the digestive enzymes level, which are more effective when the concentration of fat in the medium is higher [19].

In contrast, results of this study have pointed that bile enriched in taurocholic salts (F4) do not lead to an improved lipids digestion. Studies conducted several years ago, aimed at supplementing CF patients with taurine to achieve better digestion of fat. Literature, however, gathers controversial conclusions on this topic, some studies pointing a non-effect outcome [41,42] while others confirming its beneficial role as adjuvants [43,44]. The discrepancy may be related to different experimental designs and assessed outcomes.

Finally, another relevant finding of this study concerns the positive effect of the bile salts concentration in the digestion medium on lipolysis. The efficacy of PERT could be potentially improved if this compound was encapsulated by means of a delivery-controlled system in the duodenum in order to exert a synergic action on lipases. However, for the moment there is no other available research supporting this evidence.

A limitation of the study is inherent to the *in vitro* approach conducted. The results of *in vitro* experiments unveil mechanisms and effects but should be validated conducting clinical trials before extrapolated to in vivo conditions. These studies would be needed to confirm in terms of clinical outcomes (i.e., weight gain or coefficient of fat absorption) the reported positive effects of modifying the gastrointestinal conditions on lipids digestion.

On the other hand, the main strengths of the present work rely on the analysis of the environmental conditions being assessed from two approaches i.e. lipolysis extent and kinetics process. The statistical methods applied have a solid mathematical

background and the non-linear log-logistic dose-response functions were estimated from a high amount of data, from combination of conditions in each experiment.

In the clinical practice, thus, future combined therapeutic approaches to improve PERT would include the supplementation of enzymes with enteric-coated sodium bicarbonate, the use of PPI, and if viable, the supplementation with microcapsules of bovine bile salts, along with a high-lipid diet, achieving a synergic effect. Authors of this study strongly encourage the set-up of clinical trials aimed at confirming our findings for the later implementation of the proposed adjuvant therapies in the management of PERT.

In conclusion, our results evidence that there are gastrointestinal conditions inherent to the patients that could be modulated and, thus, strongly affect lipase activity (enzyme supplements) during dietary lipids digestion. Consequently, the main findings of the present study encourage the use of the already existing adjuvant therapies such as PPI supplementation, and can give guidance on setting-up future coadjutant therapies for PERT, or can be used as supporting references to address future clinical treatments.

ACKNOWLEDGEMENTS

The authors of this paper, on behalf of MyCyFAPP consortium, acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme for funding the project (ref. 643806).

STATEMENT OF AUTHORSHIP

All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the paper or revising it critically for important intellectual content, and, (3) final approval of the version to be submitted.

CONFLICT OF INTEREST

No conflict of interest to be declared.

STATEMENT AND FUNDING SOURCES

The study has been carried out in the framework of MyCyFAPP Project, fully funded by the European Union under Grant Agreement number 643806.

REFERENCES

1. Farrell PM (2008) The prevalence of cystic fibrosis in the European Union. J Cyst Fibros 7:450–3.
2. Sikkens ECM, Cahen DL, Kuipers EJ, Bruno MJ (2010) Pancreatic enzyme replacement therapy in chronic pancreatitis. Best Pract Res Clin Gastroenterol 24:337–47.
3. Fieker A, Philpott J, Armand M (2011) Enzyme replacement therapy for pancreatic insufficiency: Present and future. Clin Exp Gastroenterol 4:55–73.
4. Turck D, Braegger CP, Colombo C, Declercq D, Morton A, Pancheva R, et al. (2016) ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. Clin Nutr 35:557-77
5. Woestenenk JW, Ent CK Van Der, Houwen RHJ (2015) Pancreatic Enzyme Replacement Therapy and Coefficient Cystic Fibrosis. J Pediatr Gastroenterol Nutr. 61:355–60.
6. Lambert J, Agostoni C, Elmadfa I, Hulshof K, Krause E, Livingstone B, et al. (2004) Dietary intake and nutritional status of children and adolescents in Europe. Br J Nutr 92:S147-211
7. Calvo-Lerma J, Hulst JM, Asseiceira I, Claes I, Garriga M, Colombo C, et al. (2017) Nutritional status, nutrient intake and use of enzymatic supplements in paediatric patients with Cystic Fibrosis; a multicentre study with reference to current guidelines. J Cyst Fibros 16:510-518

8. Stephenson AL, Mannik LA, Walsh S, Brotherwood M, Robert R, Darling PB (2013) Longitudinal trends in nutritional status and the relation between lung function and BMI in cystic fibrosis : a population-based cohort study 1 – 3. Am J Clin Nutr 872–7.

9. E K, L V, A Z, Al. E. (2014) Factors associated with FEV1 decline in cystic fibrosis: analysis of the ECFS Patient Registry. Eur Respir J 45:125–33.

10. Li Y, Hu M, McClements DJ. (2011) Factors affecting lipase digestibility of emulsified lipids using an *in vitro* digestion model: Proposal for a standardised pH-stat method. Food Chem 126:498–505.

11. Hunter EJ. (2001) Studies on effects of dietary fatty acids as related to their position on triglycerides. Lipids 36:655–68.

12. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. (2014) A standardised static *in vitro* digestion method suitable for food – an international consensus. Food Funct Food Funct 5:1113–24.

13. Fang X, Rioux L, Labrie S, Turgeon SL (2016) Disintegration and nutrients release from cheese with different textural properties during *in vitro* digestion. FRIN 88:276-283

14. Bläckberg L, Hernell O, Bengtsson G, Olivecrona T (1979) Colipase enhances hydrolysis of dietary triglycerides in the absence of bile salts. J Clin Invest ;64:1303–8.

15. Andree M, Weber MD, Claude C, Roy MD, Claude L, Morin MD, et al. (1996) Malabsorption of bile acids in children with cystic fibrosis. N Engl J Med. 289:1001–5.

16. Hu M, Li Y, Decker EA, McClements DJ (2010) Role of calcium and calcium-binding agents on the lipase digestibility of emulsified lipids using an *in vitro* digestion model. Food Hydrocoll 24:719–25.

17. Rovner AJ, Schall JI, Mondick JT, Zhuang H, Mascarenhas MR (2014) Delayed small bowel transit in children with cystic fibrosis and pancreatic insufficiency: Commentary. Inflamm Bowel Dis Monit 14:65–6.

18. Gelfond D, Ma C, Semler J, Borowitz D (2013) Intestinal ph and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. Dig Dis Sci 58:2275–81.

19. Savary P, Iktroductios I. (1983) Specificities of lipases. J Lipid Research 4:369-384.

20. Li Y, McClements J (2010) New Mathematical Model for Interpreting pH-Stat Digestion Profiles : Impact of Lipid Droplet Characteristics on *in Vitro* Digestibility. J Agric Food Chem 58:8085–92.

21. Ozturk B, Argin S, Ozilgen M, Mcclements DJ (2015) Nanoemulsion Delivery Systems for Oil-Soluble Vitamins : Influence of Carrier Oil Type on Lipid Digestion and Vitamin D 3 Bioaccessibility. Food Chem 187:499-506

22. Fumiaki B, Park Y, Mcclements DJ (2017) Function The in fluence of lipid droplet size on the oral bioavailability of vitamin D 2 encapsulated in emulsions : an in vitro and in vivo study. Royal Society of Chemistry 8:767-777

23. Calvo-lerma J, Martinez-jimenez CP, Andrés A, Crespo-escobar P, Stav E, Schauber C, et al. (2017) Innovative approach for self-management and social welfare of children with cystic fi brosis in Europe : development , validation and implementation of an mHealth tool (MyCyFAPP). Br Med J Open. 7:e014931

24. G.Mandalari, K.Adel-Patient, V.Barkholt, C.Baro, L.Bennett, M.Bublin, et al. (2009) In vitro digestibility of β-casein and β-lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation. Regul Toxicol Pharmacol 55:372–81.

25. Armand M, Hamosh M, Philpott JR, Resnik AK, Rosenstein BJ, Hamosh A, et al. (2004) Gastric Function in Children with Cystic Fibrosis: Effect of Diet on Gastric Lipase Levels and Fat Digestion. Pediatr Res. 55:457–65.

26. Norman AP. (1979) Intestinal bile salts in cystic fibrosis. Archives of disease in childhood 54:19-24

27. Couper RTL, Corey M, Moore DJ, Fisher LJ, Forstner GG (1992) Decline of Exocrine Pancreatic Function in Cystic Fibrosis Patients with Pancreatic

Sufficiency. Pediatr Res 32:179–82.

28. Youngberg CA, Berardi RR, Howatt WF, Hyneck ML, Amidon GL, Meyer JH, et al. (1987) Comparison of gastrointestinal pH in cystic fibrosis and healthy subjects. Dig Dis Sci 32:472–80.

29. Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB, Reppas C. (2006) Characterization of the Human Upper Gastrointestinal Contents Under Conditions Simulating Bioavailability / Bioequivalence Studies 23:165-176.

30. Robinson PJ, Smith AL, Sly PD, Al RET (1990) Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. Dig Dis Sci 35:1299–304.

31. Balzola F, Cullen G, Ho GT, Russell R. Delayed small bowel transit in children with cystic fibrosis and pancreatic insufficiency: Commentary. Inflamm Bowel Dis Monit. 2014;14(2):65–6.

32. Clarke LL, Stien X, Walker NM. Intestinal bicarbonate secretion in cystic fibrosis mice. JOP. 2001;2(4):263–7.

33. Vu MK, Vecht J, Eddes EH, Biemond I, Lamers CB, Masclee AA. (1999) Antroduodenal motility in chronic pancreatitis: are abnormalities related to exocrine insufficiency? Am J Physiol Gastrointest Liver Physiol 278:G458–66.

34. Rowe SM, Heltshe SL, Gonska T, Donaldson SH, Borowitz D, Gelfond D, et al. (2014) Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. Am J Respir Crit Care Med 190:175–84.

35. Calvo-Lerma J, Martinez-Barona S, Masip E, Fornés V, Ribes-koninckx C. (2017) Pancreatic enzyme replacement therapy in cystic fibrosis: dose, variability and coefficient of fat absorption. Spanish J Gastroenterol 109:684-689

36. Durie P, Bell L, Linton W, Corey M, Forstner G (1980) Effect of cimetidine and sodium bicarbonate on pancreatic replacement therapy in cystic fibrosis. Gut. 21:778–86.

38. Proesmans M, De Boeck K. (2003) Omeprazole, a proton pump inhibitor, improves residual steatorrhoea in cystic fibrosis patients treated with high dose

pancreatic enzymes. Eur J Pediatr 162:760–3.

39. Tran TMD, Neucker A Van Den, Hendriks JJE, Forget P, Forget P. (1998) Effects of a proton-pump inhibitor in cystic fibrosis 87:553-8

40. Kalnins D, Ellis L, Corey M, Pencharz PB, Stewart C, Tullis E, et al. (2006) Enteric-coated pancreatic enzyme with bicarbonate is equal to standard enteric-coated enzyme in treating malabsorption in cystic fibrosis. J Pediatr Gastroenterol Nutr 42:256–61.

41. Thompson G, Robb T, Davison G (1987) Taurine supplementation, fat absorption and growth in cystic fibrosis. J Pediatr Gastroenterol Nutr 111:501–6.

42. Merli M, Bertasi S, Servi R, Diamanti S, Martino F, De Santis A, et al. (1994) Effect of a Medium Dose of Ursodeoxycholic Acid with or without Taurine Supplementation on the Nutritional Status of Patients with Cystic Fibrosis: A randomized, placebo-controlled, crossover trial. J Pediatr Gastroenterol nutr 19:198–203.

43. Colombo C, Arlati S, Curcio L, Maiavacca R, Garatti M. (1988) Effect of Taurine Supplementation on Fat and Bile Acid Absorption in Patients with Cystic Fibrosis 143:151-156

44. Belli D, Levy E, Darling P, Leroy C, Lepage G, Giguère R, et al. (1986) Taurine Improves the Absorption in Patients With Cystic Fibrosis. Pediatrics 80;517-23.

PAPER 6

IN VITRO DIGESTION OF LIPIDS IN REAL FOODS: INFLUENCE OF LIPID ORGANIZATION WITHIN THE FOOD MATRIX AND INTERACTIONS WITH NON-LIPID COMPONENTS

Joaquim Calvo-Lerma[a,b*], Victoria Fornés-Ferrer[b], Ana Heredia[a] and Ana Andrés[a]

[1] Universitat Politècnica de València. Instituto de Ingeniería de Alimentos para el Desarrollo. Camino de Vera s/n. 46022 Valencia (Spain). Tel. +34 961246712

[2] Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell 106. 46026 Valencia (Spain). Tel. +34 961246712

*corresponding author: joaquin_calvo@iislafe.es

ABSTRACT

In vitro digestion research has scarcely addressed the assessment of the complexity of digestion in real food. The aim of the present study was to evaluate the influence of intestinal conditions, non-lipid components, and lipid organization within the food matrix on lipolysis extent. A selection of 52 foods was studied under different simulated intestinal conditions. Linear mixed regression models were applied to explain associations of food properties with lipolysis. Normal intestinal conditions allowed for optimal lipolysis in most of the foods in contrast to the altered intestinal scenario (30 vs 1 food reaching >90% lipolysis). Lipid-protein and lipid-starch interactions were evidenced to significantly affect lipolysis ($p<0.001$) in all the digestion conditions, decreasing in those foods with low fat and high protein or high starch content. In addition, under decreased intestinal pH and bile concentration, lipolysis was lower in foods with complex solid structures and continuous lipid phase than in the oil-in-water continuous aqueous phase (global $p <0.01$). However, in the normal conditions lipid organization within the food matrix did not show a significant effect on lipolysis (global p 0.08). In conclusion, food properties play a crucial role in lipolysis, which should be considered when establishing dietary recommendations.

KEYWORDS: *in vitro* digestion, lipolysis, nutrition, pancreatic insufficiency, food matrix

PRACTICAL APPLICATION

The impact of food composition and lipid structure within the food matrix on lipolysis suppose key results in the development of an evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis. These results have contributed to the development of a mobile app for these patients, which includes an algorithm for enzyme dose prediction based on food properties. The app is currently being tested in clinical trial setting. Besides, results may contribute to dietary recommendations to the general population and to the development of new foods.

1. INTRODUCTION

In vitro digestion methods rose in the past years as a powerful approach to study several aspects related to foods biotransformation within the gastrointestinal tract, especially those related to luminal digestion. They are a useful tool for reproducing the process in lab, under controlled, accurate and reproducible conditions. In this sense, the internationally harmonised protocol of Minekus et al. (2014) set up a common framework to conduct static *in vitro* digestion studies, indicating that the pertinent amendments have to be applied according to the nature of the research (Minekus et al., 2014).

Then on, numerous studies have addressed the study of food properties on bioaccessibility of bioactive compounds, and the hydrolysis of macronutrients, among others. The vast majority of these studies have focused on monocomponent systems or ideal emulsions, in order to simplify the multifactor and complex nature of foods under digestion and to drive solid conclusions. Therefore, there are still few studies in the literature addressing the complexity of the real food and the influence of different variables in the gastrointestinal tract (Guo, Ye, Bellissimo, Singh, & Rousseau, 2017). Nonetheless, there is sufficient evidence to state that within the complexity of food structures, multiple matrix interactions occur among the components conforming it,

which unequivocally alter the observed behaviour of the components when assessed in isolation. However, today there is a poor systematic understanding of the impact of the food matrix on the processes that occur during nutrient digestion (Guo et al., 2017; Taylor et al., 2009).

One of the utility of studying the digestion of real foods is the potential application of the generated knowledge on the improvement of a food-related health condition. Exocrine Pancreatic Insufficiency (EPI) which is associated to some diseases as for example Cystic Fibrosis (CF) (Woestenenk, van der Ent, & Houwen, 2015), is associated to altered intestinal conditions, including a lower duodenal pH and up to 10 times lower bile salts concentration as compared to healthy individuals (Gelfond, Ma, Semler, & Borowitz, 2013; Harries et al., 1979; Robinson, Smith, & Sly, 1990). Thus, they have to adhere to pancreatic enzyme replacement therapy (PERT) to palliate this disorder, consisting on the exogenous administration of pancreatic enzymes in every meal. It enables digestion of nutrients, especially lipids, which is the most compromised. However, a PERT dosing criterion stills lacking of scientific evidence that considers the variety and complex nature of foods, being the general recommendation 2000-4000 Lipase Units / g lipid (LU/g) (Turck et al., 2016). In this sense, the current need of knowledge of lipolysis in real foods(Fieker, Philpott, & Armand, 2011; Li, Hu, & McClements, 2011) is an ideal target for applying the *in vitro* digestion methodology to adjust PERT.

Over the years, several studies in CF patients have been unable to establish any association between the dose of PERT and the coefficient of fat absorption, the method used to assess the PERT dose adjustment. Thus, we can find several authors claiming for an evidence-based method to adjust the dosing criterion in this therapy (Borowitz et al., 2005; Fieker et al., 2011; Schall, Bentley, & Stallings, 2006). In none of the studies assessing PERT dose on lipid digestion, composition of foods has been considered, so our hypothesis is that food properties could impact on PERT efficacy, thus explaining the historical lack of association between PERT dose and clinical outcomes.

The European Union's Horizon 2020 programme for research and innovation has prioritised research for tackling societal challenges (European Commission, 2015). Following this practical approach, MyCyFAPP project pursues the establishment of a valid method to adjust PERT (Calvo-Lerma et al., 2017). As part of this project, the aim of the present study was to analyse lipolysis of a wide range of real foods under altered simulated gastrointestinal conditions in order to explain the influence of the inherent-to-food properties, such as nutrient composition and their interactions on lipolysis, and the lipid organization within the food matrix.

2. METHODS

2.1. Materials

The selection of foods to be *in vitro* digested (n =52) was made on the basis of a European multicentre study on CF nutritional habits and covering the whole range of food products (Calvo-Lerma et al., 2018): dairy, meat, fish, egg, nuts, fruit, oils, fats, potato, sweets and cereal. Nutritional information of foods was collected from the official composition database of EuroFIR®. The lipid organization of the foods was established according to the criterion posed by Michalski et al. (Michalski et al., 2013). Information of the characteristics of the study foods can be found in **Annex 5**.

Pancreatic enzyme supplements (Kreon® 10000 LU) were kindly donated by Hospital Universitari i Politècnic La Fe (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme in the shape of gastro-resistant microspheres equivalent to 10000 lipase U, 8000 amylase U, and 600 protease U.

For the preparation of the simulated digestive fluids, the following chemicals were needed: pepsin from porcine gastric mucosa (≥2500 U / g protein), bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$ and $CaCl_2$ all of them from Sigma-Aldrich Chemical Company (St Louis, MO, USA). NaOH (1 N) and HCl (1 N), were acquired from *AppliChem Panreac*. For the analytical determinations, Triton-X 100 %, and the analytical standard palmitic acid were acquired from *Sigma-Aldrich*.

2.2. Experimental design

The experimental design consisted of a set of *in vitro* digestions with a dose of enzymes fixed at 2000 LU/g of lipid and different combinations of intestinal pH and bile salts concentration: pH6/10mM, pH7/1mM and pH7/10mM, as study variables in order to analyse the impact of altered intestinal environments on lipolysis. Of note, the combination pH6/1mM would represents the worst possible case scenario in CF (Gelfond et al., 2013; Harries et al., 1979; Robinson et al., 1990) and the pH7/10mM approaches the regular intestinal conditions of a healthy adult (Minekus et al., 2014). All the experiments were conducted in triplicate, resulting in a total of 624 *in vitro* digestions.

2.3. *In vitro* digestion process

Food samples (5 g) were placed into 50 mL falcon tubes and *in vitro* digested according to the above-mentioned experimental design. The static *in vitro* protocol applied consisted on three different stages, oral, gastric and intestinal, established by the cost action-INFOGEST and published by Minekus et al. (Minekus, et al. ,2014). The digestion fluids were prepared fresh daily from stock solutions (**Annex 3**), and the enzymatic activity of the solutions was tested before each experiment following the protocol proposed by Carrière et al. (Carrière et al., 2000).

Oral stage: Simulated salivary fluid (5 mL) (SSF; pH 8) at 37 ºC, was added to the food sample in a ratio 1:1 (v/w) and in case of solid foods, properly homogenized with a kitchen blender for 3 minutes (Vario Mixer, Ufesa 600 W).

Gastric stage: After the oral stage, simulated gastric fluid (SGF; pH 3) was added to each tube containing the oral bolus (1:1 v/w). Pepsin was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). At this point PERT dose 2000 LU/g lipid was added in order to simulate the oral administration of the enzymatic supplement, which is resistant to gastric digestion and the release of the enzymes occurs at the intestinal stage. The pH of the mixtures was adjusted with HCl (1N) to pH 2.8 ± 0.1 and samples were rotated head- over-heels at 55 rpm for 2 h at 37 ºC using

an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and an incubator chamber Selecta (JP Selecta SA, Barcelona). These mixing conditions provided constant mechanical energy to induce the breakdown of the food matrix during digestion.

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF; pH 6 or 7) containing the bile salts (concentration 1 or 10 mM) was added in a proportion 1:1 (v/w) to each tube containing the gastric chime. The pH of the mixtures was adjusted to pH 6.0 ± 0.1 or 7 ± 0.1, depending on the experimental design, with NaOH (1N). Samples were then rotated head-over-heels at 55 rpm for another 2 h at 37 ºC. pH was monitored during the digestion process and readjusted if necessary as lipase is not active at pH below 5.7 (Lesmes & McClements, 2012). After 2 hours of intestinal digestion samples were placed in ice and pH adjusted to 9 to ionize all the free fatty acids and stop lipase activity (González-Bacerio, Rodríguez Hernández, & del Monte Martínez, 2010).

2.4. Analytical determinations

Lipids digestion was determined by measuring the free fatty acids (FFA) released after the *in vitro* gastrointestinal digestion process. Micellar phase from digested samples (100 µl) was separated by decantation using a 1.4 mm sieve and mixed with 10 mL of a solution made of 5.6 % Triton X-100 and 6 % ethanol in water, to solubilize the free fatty acids. The release of free fatty acids after digestion was measured on the diluted samples using a free fatty acid spectrophotometric assay kit (Roche Diagnostics, Indianapolis, IN, USA) in a spectrophotometer (UV/vis, Beckman Coulter) (Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014). Palmitic acid standard was used for quantitative determination of FFA. Lipolysis extent was estimated assuming the release of 2 moles of fatty acids per 1 mole of triglycerides (Lamothe et al., 2014).

2.5. Statistical analysis

The variables included for the statistical analysis were the nutritional information of food products: energy, protein, total carbohydrates, starch, sugar, total lipids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), fibre, calcium, iron and sodium; and the lipid structure in the food matrix (Michalski et al., 2013): complex solid structure (lipid inclusion in CH and protein matrix and lipid inclusion in protein matrix), continuous aqueous phase (intracellular lipid droplets and tissues and oil-in-water emulsions) and continuous lipid phase (free fat, particles in solid fat and water-in-oil emulsions). The response variable was lipolysis extent (%).

Data were summarised using mean, standard deviation, median and 1st and 3rd quartile in the case of continuous variables and with absolute ant relative frequencies in the case of categorical variables.

A heatmap diagram was performed to represent the similarity between all the assessed foods at the different experimental conditions. The distance measure used for heatmap clustering was Euclidean and the clustering method was Complete.

Linear mixed regression models were performed to assess the effect of the food composition on the lipolysis extent and other factors such as lipid organization in the food matrix were included as covariates. Additionally, because observations of the same food are more likely to have similar lipolysis extent due to their nutritional characteristics, the linear regression models were extended with the "Food" variable as random effect with random intercept to correct for the no independence of the data.

All analyses were performed using software R (version 3.4.2) using packages betareg (version 3.1-0), lme4 (version 1.1-14) and NMF (version 0.20.6). A p-value lower than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effect of the intestinal conditions on lipolysis extent

The selection of 52 foods was *in vitro* digested at different intestinal conditions.

When representing lipolysis extent of all the assessed foods as a function of the intestinal conditions (**Figure 4.13**), the condition of intestinal pH7 and bile 10mM resulted to be the most related with a higher lipolysis extent, finding 30 foods with lipolysis extent >90%. In contrast, the combination of pH6 and bile 1mM depicted the lowest lipolysis results being 1 food reaching >90% lipolysis. This is in accordance to previous research by our group (Calvo-Lerma et al., 2018). The mechanisms underpinning this result is that pancreatic lipase is well known to have a higher activity at pH 7 than 6 (Robinson et al., 1990). In addition, bile salts contribute to the emulsification process of lipids in the digestive fluids and consequently increasing the interfacial surface of the lipids available for being hydrolysed (Verger & De Haas, 1976); therefore the higher the bile concentration the higher the lipolysis is enabled.

However, the intermediate conditions, i.e. pH6 bile 10mM and pH7 bile 1 mM, showed different responses to the lipolysis (10 and 11 foods with >90% lipolysis respectively), due to the complexity of the matrices being disintegrated at different rates and their interactions with the gastrointestinal environment. The heatmap brings a picture where it is easy to observe that the extension of lipolysis is more pH dependent for some foods than for others, as it is the case of cured cheese, spreadable chocolate or crunchy biscuit bars, while in others bile concentration is the main factor affecting fat hydrolysis (butter, drumstick, boiled egg or bread).

Additionally, there are some foods for which lipolysis depends on both factors, the pH and bile concentration (i.e. Frankfurt sausage). Maldonado-Valderrama et al. 2011, reported that there is a specific interaction between bile salts and co-lipase to promote lipase activity (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011).

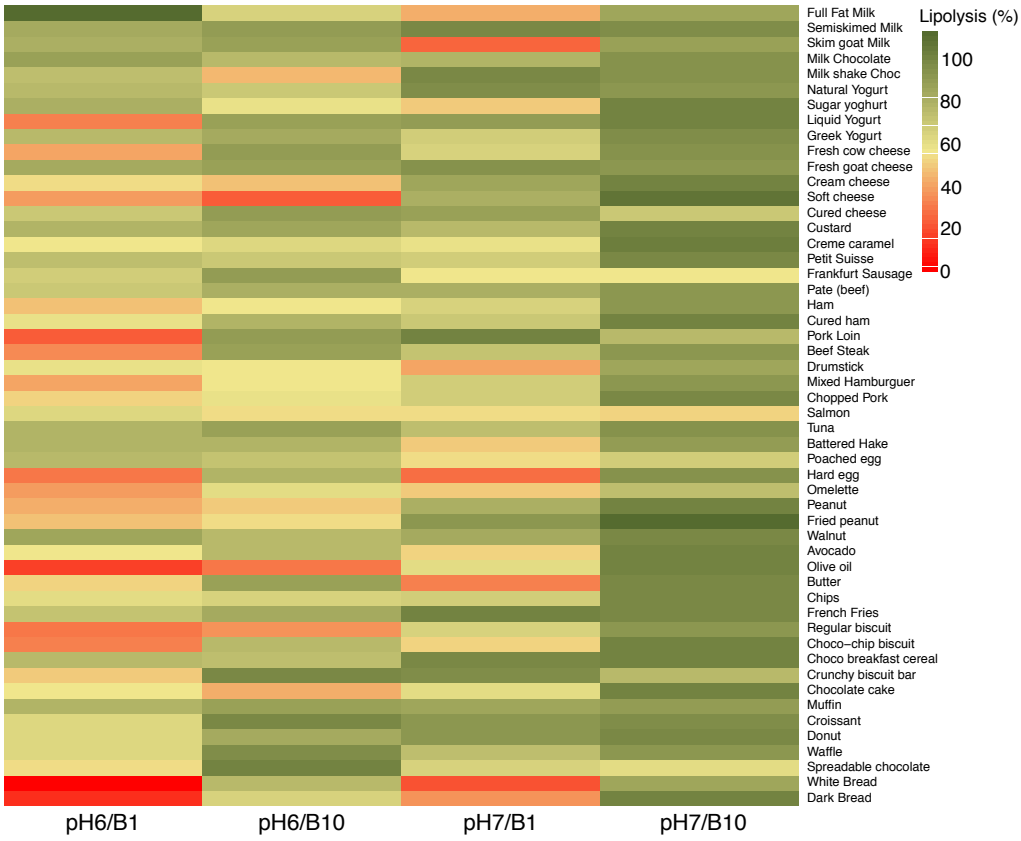


Figure 4.13. Heatmap representing lipolysis extent of the 52 assessed foods under different combinations of intestinal conditions pH/bile concentration at the PERT dose of 2000 LU/g fat

Bile salts play a very important role in emulsifying fats entering the small intestine in chyme but also the interfacial composition of proteins and polysaccharides of emulsified fat. The consequence or the response to all these coupled factors is evidenced by differences on percentage of fat digested at the end of the intestinal stage. Therefore, for some foods the bile concentration showed a more favouring role than the pH such as in the hard egg or spreadable chocolate, whilst in others the opposite response was found, like in milk. Besides the influence of interfacial composition digestion depends on the size of the emulsion droplets in the small intestine, since this influences the amount of lipid surface area exposed to the lipase, and the droplet size is inherent to the rheological properties of the surrounding

medium (Taylor et al., 2009). As a consequence, different foods can result in a wide variety of intestinal environments, where the presence of bile salts may either promote or inhibit the activity of pancreatic lipase depending on their concentration (Bauer, Jakob, & Mosenthin, 2005; Lowe, 2002).

Bile salts can promote or inhibit lipase activity, depending to their ability to solubilize lipid digestion products and remove them from the oil-water interface. This variable effect will depend on the type of lipids in the digestion medium (lipid organization and capacity of the lipids to be released from the matrix) and then, on the capacity of the bile salts to compete for the oil-water interface with the lipase (Gargouri, Julien, Bois, Verger, & Sarda, 1983). The result is that lipids accessibility in food matrix and the interactions of the non-lipid components of the matrix remarkably contribute to modulate lipids digestion. These interactions were analysed from the experimental data obtained in this work and the results are shown in the next section.

3.2. Interactions of lipids with the non-lipid components of the same food matrix

When analysing the effect of nutrient composition on lipolysis in a multivariable setting, no clear effects were obtained, i.e. the amount of none of the nutrients was significantly associated with lipolysis extent. This can be interpreted as the presence of a specific nutrient could favour lipid digestion in some foods but diminish it in some others (Guo et al., 2017) being the nutrient concentrations and the interactions among nutrients the impact factor on lipolysis extent. Foods are complex structures in which lipids can be present at different levels of interaction with the other components of the matrix. In this sense, in solid matrices lipids can be embedded within hydrogel or protein structures, like in cheese, meat, fish or nuts. In contrast, in liquid matrices like milk, lipids are less bounded to the structure what make them easy accessible to the enzyme action. Thus, digestion of lipids may depend on the breakdown of these matrices before they can be exposed to lipases (Chen, Remondetto, & Subirade, 2006). Besides, many of these non-lipid components may interfere with lipid digestion by altering the viscosity of the digestion media, by

competing with lipase for the oil-water interface of the emulsified lipids, or by interfering with enzyme activity(Taylor et al., 2009). Therefore, the statistical models were readjusted considering these interactions among macronutrients (**Table 4.12**).

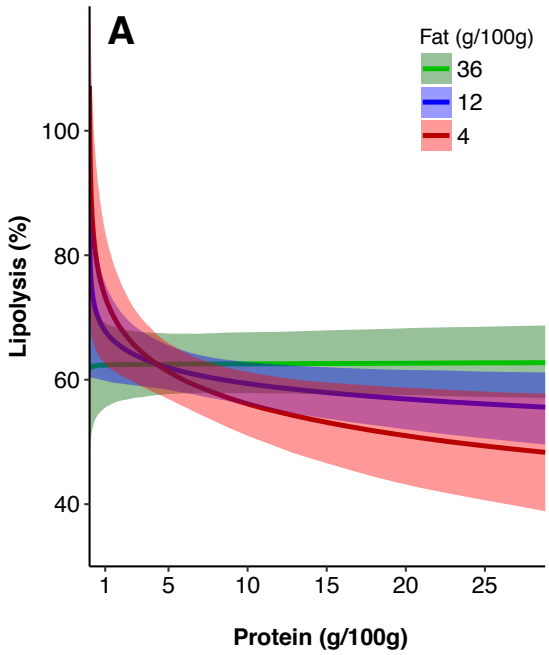
Table 4.12. Linear mixed regression models showing the influence of nutritional composition on lipolysis: interaction between protein and lipids, interaction of type of fatty acid with the protein content and the interaction between starch content and lipids

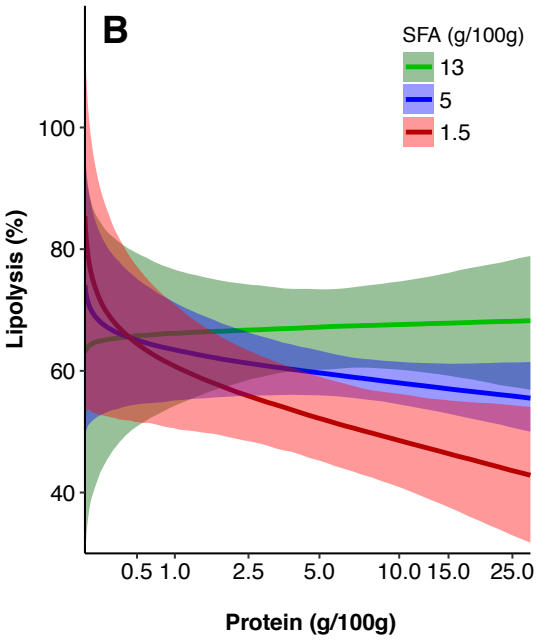
Interaction	Estimate	95% Confidence interval	p-value
Protein : lipids	3.431	[1.491, 5.371]	<0.001
Protein : SFA	6.141	[1.48, 10.825]	0.027
Protein : MUFA	-2.972	[-7.781, 2.043]	0.295
Protein : PUFA	5.09	[-2.222, 12.03]	0.217
Starch : lipids	4.031	[2.195, 5.868]	<0.001

The statistical analysis revealed that the interaction of protein content with lipids content (CI 95% [1.49, 5.37] p<0.001) and with saturated fatty acids (SFA) (CI 95% [1.50, 10.85] p 0.027) had a significant effect on lipolysis extent, and also the interaction between starch content and lipids concentration (CI 95% [2.20, 5.87] p <0.001).

The interaction protein-lipids (**Figure 4.14a**) did not represent a change in lipolysis extent in those foods with a high amount of fat (around 36g/100g) regardless the content of protein. According to this, cured cheese and chips, which both contain 34g fat/100g product but very different content of protein (24 and 6g/100g respectively) showed a similar lipolysis extent. However, foods with a medium (12g/100g) and low (4g/100g) content of lipids resulted in a decreased lipolysis according to the increasing protein content. For example tuna, which is low in fat (4g/100g) but has as much protein content as cheese (around 25g/100g), showed a

much lower lipolysis extent, i.e. 60 vs 80 %, when digested under the conditions of pH6 bile 1mM.





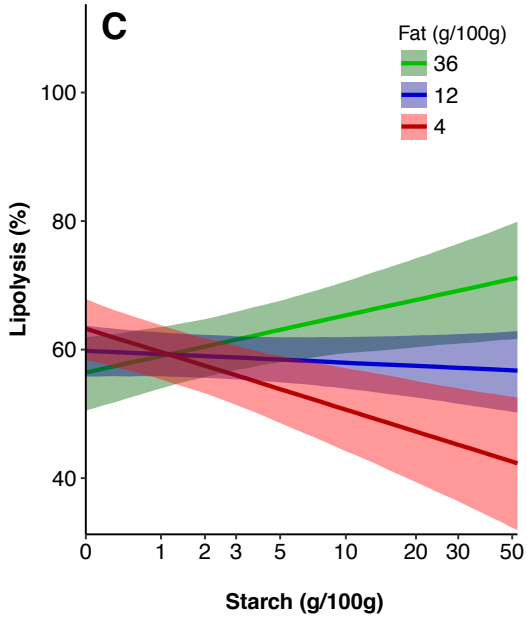


Figure 4.14. Nutrient interaction and the effect on lipolysis: protein-lipid interaction (a), protein-SFA interaction (b) and starch-lipid interaction (c). *SFA, saturated fatty acids*

The interaction protein-SFA (**Figure 4.14b**) showed a similar effect than the protein-lipid. Foods with a medium and low SFA content tended to decrease lipolysis extent with the increasing protein content, whereas in this case, the high SFA content led to a mild increase lipolysis with the increasing protein content. The physical state of the food matrix and lipids process have proved to play a crucial role during digestion, and the composition in fatty acids is an indirect measure of the fat melting point (Knothe & Dunn, 2009). In front of the difficulty of assigning a specific melting temperature to all the foods/fats, the saturated/unsaturated fatty acids ratio was considered. The statistical analysis revealed a significant association between this ratio and lipolysis extent (p=0.002, CI= [2.526, 10.185]), what meant that the higher the SFA content and the lower MUFA and PUFA, the higher lipolysis. This finding suggests that in those foods with a saturated fatty acid profile, and so a higher lipids melting point, lipolysis is more favourable.

The observed interaction between the content of proteins and lipids has not been explicitly reported in the literature, although one study attributed the amount of protein forming a gel mesh to the lipolysis achieved of a lipid emulsion. In this study the higher the gel structure forming capacity of the protein was related to the lower lipase diffusion capacity to the interface of fat droplets. As proteases facilitated the breakdown of the gel network, digestion of fat occurred (Sarkar et al., 2015). Therefore, the lipid-protein interaction of our study could be explained in a physicochemical basis as a similar phenomenon as the one described by these authors.

Among the rest of the nutrients, only starch was found to have a significant effect on lipolysis **(Figure 4.14c)**; total carbohydrates, sugar, fibre, calcium, iron and sodium did not show any effect on lipolysis extent, neither when studying their interaction with the macronutrients. The higher the content of starch, the lower lipolysis in foods with a low content of lipids, while the inverse association was obtained for foods with a high content of lipids. According to the model, a high-fat low-starch product like pate, would show a higher lipolysis than a food with a similar content of starch but with a low content of lipids like bread. These results are in accordance with those reported by other authors: Nakamura et al. (2006) concluded that polysaccharides protect the lipid emulsion droplet surface by forming a thick hydrated layer (Nakamura, Yoshida, Maeda, & M. Corredig, 2006). It has been proposed that carbohydrates can affect lipid digestion in the small intestine through a variety of physicochemical mechanisms, such as increasing viscosity of the digestion medium, alteration of droplet disruption or coalescence kinetics, binding of bile salts, phospholipids, or enzymes (Guo et al., 2017; Lairon, 1997; Lairon, Play, & Jourdheuil-Rahmani, 2007). This evidence suggests that in the case of the assessed breads which had 40 and 50 g starch /100g of product, lipolysis could be low due to the viscosity these high amounts may have conferred to the digestion medium. In addition, as compared to other high-starch products such as biscuits ranging in starch content from 35 to 52g/100g and in lipids from 11 to 22g/100g, lipolysis was much lower in breads due to their low content of lipids, 4.4 and 1.5g/100g.

Overall, the study of the interaction of lipids with protein and starch, which play a key role in food structure, revealed that lipolysis is decreased in those foods with low lipid and high content of the other macronutrients, while this effect is not shown if the food has a high lipid content too. Besides the discussed mechanisms supporting these results, Binks et al. (2002) pointed that solid particles like carbohydrates and protein, can anchor irreversibly to the oil-water interface, limiting the ability of bile salts and enzymes to physically contact, thus decreasing lipolysis. The effect of the food structure, especially lipid structure, is further studied in the following section.

3.3. Organization of lipids in food matrices and their influence on lipolysis

Besides nutrient composition, the lipid organization in the food matrix was considered as a categorical variable to explore its influence on lipolysis. The 52 tested foods were classified in three groups: 1) foods with complex solid structure, 2) foods with a continuous aqueous phase and 3) foods with a continuous lipid phase. Foods with complex solid structure were divided in two subgroups, those with lipids inclusion in a protein matrix (e.g. cheese) and those with lipids inclusion in carbohydrate and protein matrix (e.g. cookies). Lipids in foods with a continuous aqueous phase can be structure as oil in water emulsion (e.g. milk) or as intracellular lipid droplets and membrane structures (tissues) (e.g. meat, egg yolk, vegetables). Finally, different subgroups of foods with a continuous lipid phase were established: free fat (e.g. oil, lard), foods with particles dispersed in solid fat (e.g. chocolate) and water in oil emulsions (e.g. butter or margarine). For the analysis of the results, the two intestinal digestion conditions were explored separately: intestinal pH7 and bile concentration 10 mM (corresponding to the standard conditions of a healthy adult), and pH6 bile 1mM corresponding to exocrine pancreatic insufficiency. **Table 4.13** and **Table 4.14** show the results of the linear mixed regression model for each set of conditions, in which the differences in lipolysis for the lipid structures and substructures are reported as compared to the oil-in-water emulsion substructure as the reference.

When digested under the conditions intestinal pH6 and bile concentration 1mM (**Table 13**), lipolysis extent resulted significantly different among lipid structures as compared to the oil-in-water emulsion structure (global p 0.01), which showed the highest lipolysis extent (**Figure 4.15**). The reason for foods with lipids structured as oil-in-water emulsion showing the highest lipolysis extent, might be due to the presence of surfactant agents which are inherently present, what at the time increase the lipid droplets surface area (Verger & De Haas, 1976). In addition, liquid phase systems offer less resistance to diffusion, so enzymes accessibility to fat is facilitated (Guo et al., 2017). Food emulsions, such as milk, cream and dairy-based deserts may be stabilized by a wide variety of different natural or added emulsifiers, including small molecule surfactants, phospholipids, proteins, polysaccharide, and their mixtures (McClements, 2005), what make lipid droplets more accessible to the enzymes.

Table 4.13. Linear mixed regression model explaining the effect of the lipid structure in the food matrix on lipolysis at the digestion conditions of intestinal pH6 and bile concentration 1mM

Structure	Sub-structure	Estimate	Std. Error	95% Confidence Interval		P-value
complex solid structure	lipid inclusion in protein matrix	-17.167	8.753	[-33.43	-1.21]	0.050
complex solid structure	lipid inclusion in CH and protein matrix	-24.468	8.081	[-39.48	-9.46]	0.004
continuous aqueous phase	intracellular lipid droplets and tissues	-21.482	7.649	[-35.69	-7.27]	0.007
continuous lipid phase	free fat	-57.281	20.947	[-96.19	-18.37]	0.009

continuous lipid phase	particles in solid fat	-20.027	20.947	[-58.94	18.88]	0.344
continuous lipid phase	water-in-oil emulsion	-23.157	20.947	[-62.07	15.75]	0.275
Global						0.01

Table 4.14. Linear mixed regression model explaining the effect of the lipid structure in the food matrix on lipolysis at the digestion conditions of intestinal pH7 and bile concentration 10mM

Structure	Sub-structure	Estimate	Std. Error	95% Confidence Interval	P-value
complex solid structure	lipid inclusion in protein matrix	-6.976	5.16	[-16.56, 2.60]	0.183
complex solid structure	lipid inclusion in CH and protein matrix	-2.692	4.76	[-11.54, 6.15]	0.575
continuous aqueous phase	intracellular lipid droplets and tissues	-7.358	4.51	[-15.73, 1.01]	0.11
continuous lipid phase	free fat	4.109	12.34	[-18.89, 27.0]	0.741
continuous lipid phase	particles in solid fat	-35.66	12.34	[-58.6, -12.73]	0.006
continuous lipid phase	water-in-oil emulsion	2.861	12.34	[-20.1, 25.79]	0.818
Global					0.08

In this scenario, the free fat structure, i.e. olive oil, showed the most negative effect on lipolysis extent (95% CI [-96.2, -18.4] p 0.009). Oil has 98% lipid composition and contains no other macronutrient such as protein or starch. This makes it a bulky

substrate for the enzymes, with no other surfactant in the medium than a low concentration of bile salts (1mM), what represent unfavourable conditions for lipase activity. The foods with complex solid structures did also achieve a significant lower lipolysis. The negative effect of these matrices was more pronounced in the case of solid matrices containing both protein and carbohydrates, such as bread, biscuits and pastries (95% CI [-39.5, -9.5] p 0.004) than in those containing mainly protein, including meat and fish (95% CI [-33.4, -1.2]. The reason for the difference between the two sub-structures may relay on the starch content of some foods such as bread, which, as above discussed, may increase the viscosity of the digestion medium, making difficult the accessibility of lipase to fat (Guo et al., 2017). The other lipid sub-structure included in the continuous aqueous phase category, i.e. intracellular lipid droplets and membrane structures, was also negatively associated to lipolysis (95% CI [-39.7, -7.3] p 0.007). In this type of structure, lipids are very embedded like in the case of nuts what makes difficult the matrix disruption during digestion and therefore the release of fat to be accessible to the enzymes (Grundy et al., 2016).

In contrast, when assessing lipid organization in the digestion conditions of intestinal pH7 and bile concentration 10mM, non-significant effect on lipolysis was observed except in the case of “particles in solid fat” (**Table 14**), represented by chocolate products, in which lipolysis was negatively associated to this structure (95% CI [-58.6, -12.7] p 0.006). Thus, lipids in chocolate products were those more difficultly hydrolysed in both digestion environments scenarios assessed. Apart from this exception, all the categories obtained a similar median lipolysis extent between 90 and 100% (**Figure 4.16**).

This finding evidences that, when in normal digestion conditions, the lipid organization in the food matrix does not affect lipolysis, and satisfactory extents are achieved. It is of special relevance in the case of Cystic Fibrosis, in which pH and bile salts concentration are lower, and in which the dose of enzymatic supplements to be taken will depend on this factor. For example, according to our results, in the intestinal pH6 and bile 1mM scenario, for some dairy products the assessed dose of 2000 LU/g

fat would enable for optimal lipolysis >90%. However, in the case of foods with other structures, higher doses of the supplements might be given in order to reach higher lipolysis extents.

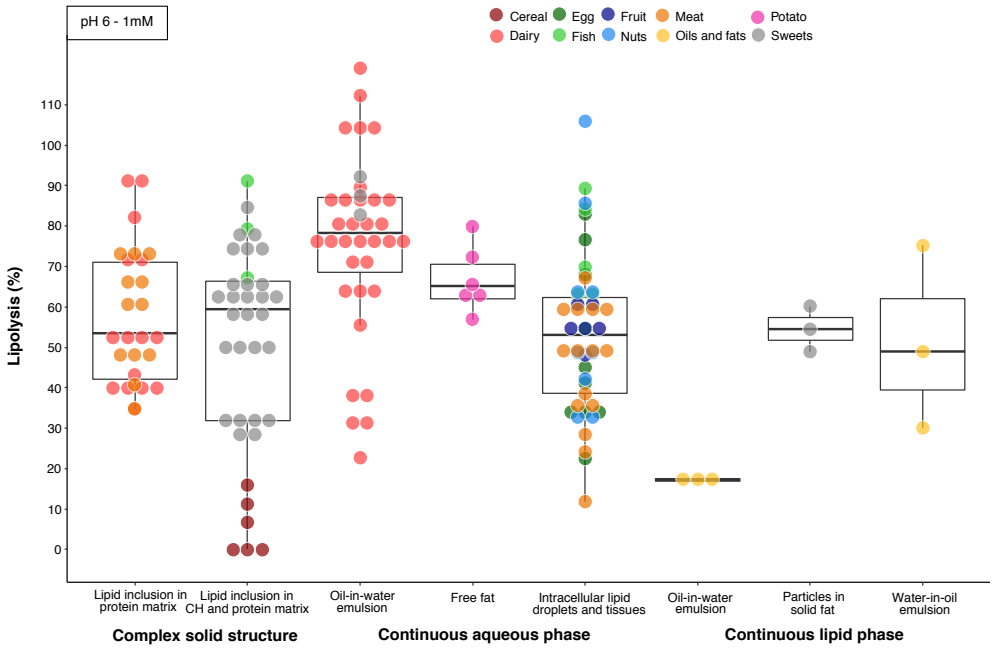


Figure 4.15. Boxplots representing lipolysis extent of the assessed food groups according to the lipid structure in the food matrix, under the digestion conditions of intestinal pH6 and bile salts concentration 1mM

To sum up, through the present study we have elucidated the role of different factors on lipolysis in real foods. First, the intestinal conditions play a key role, being the conditions pH7 and bile concentration 10mM those leading to lipolysis extents >90% in almost all the assessed foods. Second, the results revealed strong interactions between nutrients conditioning lipolysis: foods with medium and low lipid (and SFA) content showed decreased lipolysis extent when the content of protein or starch was high, but not those with high amount of fat. Finally, in the intestinal conditions pH6 and bile 1mM, the different type of lipid organization in the food matrix is significantly

associated with a lower lipolysis as compared to the oil-in-water emulsions, which showed the highest median value.

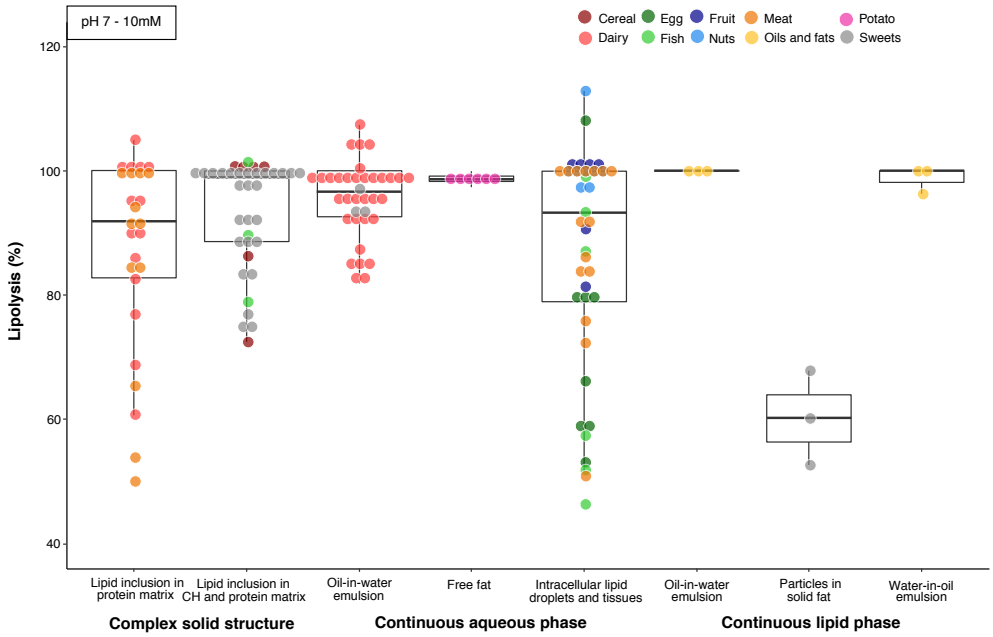


Figure 4.16. Boxplots representing lipolysis extent of the assessed food groups according to the lipid structure in the food matrix, under the digestion conditions of intestinal pH7 and bile salts concentration 10mM

4. CONCLUSION

Besides simulated intestinal conditions, food properties such as nutrients composition and their interactions, and lipid organization in the food matrix determine lipolysis during *in vitro* digestion of real foods, leading to a wide range of lipolysis extents. Therefore, food characteristics should be considered for dietary recommendations whatever the objective is to maximize or minimize lipid extent, but especially for people suffering EPI. The results of this study will be used as key data in the development of a predictive enzyme dose calculator algorithm in the framework of MyCyFAPP Project, which will support cystic fibrosis patients self-adjusting of the dose based on evidence-based data.

ACKNOWLEDGEMENTS

Authors of this paper acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme (PHC-26-2014 call Self-management of health and disease: citizen engagement and mHealth) for fully funding this research under grant agreement number 643806.

AUTHOR CONTRIBUTIONS

J. Calvo-Lerma and A. Andrés designed the study. J. Calvo-Lerma collected the data. V. Fornés-Ferrer and J. Calvo-Lerma performed the statistical analysis. J. Calvo-Lerma, A. Andrés and A. Heredia interpreted the results and drafted the manuscript. All the authors reviewed the manuscript and approved its final version for submission.

5. REFERENCES

Bauer, E., Jakob, S., & Mosenthin, R. (2005). Principles of physiology of lipid digestion. *Asian-Australasian Journal of Animal Sciences*, *18*(2), 282–295.

Borowitz, D., Durie, P. R., Clarke, L. L., Werlin, S. L., Taylor, C. J., Semler, J., & Baker, R. D. (2005). Gastrointestinal outcomes and confounders in cystic fibrosis. *Journal of Pediatric Gastroenterology and Nutrition*, *41*(3), 273–285.

Calvo-Lerma, J., Martinez-Jimenez, C. P., Lázaro-Ramos, J. P., Andrés, A., Crespo-Escobar, P., Stav, E., ., & Colombo, C. (2017). Innovative approach for self-management and social welfare of children with cystic fibrosis in Europe: development, validation and implementation of an mHealth tool (MyCyFAPP). *British Medical Journal Open*, *7*(3), e014931.

Calvo-Lerma, J., Hulst, J., Boon, M., Martins, T., Garriga, M., ., & Ribes-Koninckx, C. (2018) Children with Cystic Fibrosis present with dietary imbalances: A European multicentre comparison of food groups and origin of nutrient intake. *Journal of the Academy of Nutrition and Dietetics. (under review).*

Calvo-Lerma, J., Fornés-Ferrer V., Heredia, A. & Andrés, A. (2018) The role of

gastrointestinal conditions on *in vitro* lipids' digestion. *Clinical Nutrition (under review)*

Carrière, F., Renou, C., Lopez, V., De Caro, J., Ferrato, F., Lengsfeld, H., & Verger, R. (2000). The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test meals. *Gastroenterology*, *119*(4), 949–960.

Chen, L., Remondetto, G. E., & Subirade, M. (2006). Food protein-based materials as nutraceutical delivery systems. *Trends in Food Science & Technology*, *17*(5), 272–283.

EUROPEAN COMMISSION. (2015). Horizon 2020. Work Programme 2014-2015. Health, Demographic change and wellbeing.

Fieker, A., Philpott, J., & Armand, M. (2011). Enzyme replacement therapy for pancreatic insufficiency: present and future. *Clinical and Experimental Gastroenterology*, *4*(4), 55–73.

Gargouri, Y., Julien, R., Bois, A. G., Verger, R., & Sarda, L. (1983). Studies on the detergent inhibition of pancreatic lipase activity. *Journal of Lipid Research*, *24*(10), 1336–1342.

Gelfond, D., Ma, C., Semler, J., & Borowitz, D. (2013). Intestinal pH and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. *Digestive Diseases and Sciences*, *58*(8), 2275–2281.

González-Bacerio, J., Rodríguez Hernández, J., & del Monte Martínez, A. (2010). Lipases: enzymes with potential for the development of immobilized biocatalysts by interfacial adsorption. *Rev. Colomb. Biotecnol.*, *12*, 113–140.

Grundy, M. M. L., Carriere, F., Mackie, A. R., Gray, D. A., Butterworth, P. J., & P.R. Ellis. (2016). The role of plant cell wall encapsulation and porosity in regulating lipolysis during the digestion of almond seeds. *Food & function2*, *7*(1), 69–78.

Guo, Q., Ye, A., Bellissimo, N., Singh, H., & Rousseau, D. (2017). Modulating fat digestion through food structure design. *Progress in Lipid Research*, *68*, 109–118.

Harries, J. T., Muller, D. P., McCollum, J. P., Lipson, A., Roma, E., & Norman, A. P. (1979). Intestinal bile salts in cystic fibrosis: studies in the patient and experimental

animal. *Archives of Disease in Childhood*, *54*(1), 19–24.

Knothe, G., & Dunn, R. O. (2009). A comprehensive evaluation of the melting points of fatty acids and esters determined by differential scanning calorimetry. *Journal of the American Oil Chemists' Society*, *89*(9), 843–856.

Lairon, D. (1997). Soluble fibers and dietary lipids. In Dietary fiber in health and disease (pp.). Springer, Boston, MA. In *Dietary fiber in health and disease* (pp. 99–108).

Lairon, D., Play, B., & Jourdheuil-Rahmani, D. (2007). Digestible and indigestible carbohydrates: interactions with postprandial lipid metabolism. *The Journal of Nutritional Biochemistry*, *18*(4), 217–227.

Lamothe, S., Azimy, N., Bazinet, L., Couillard, C., & Britten, M. (2014). Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment. *Food & Function*, *5*(10), 2621–2631.

Lesmes, U., & McClements, D. J. (2012). Controlling lipid digestibility: Response of lipid droplets coated by β-lactoglobulin-dextran Maillard conjugates to simulated gastrointestinal conditions. *Food Hydrocolloids*, *26*, 221–230.

Li, Y., Hu, M., & McClements, D. J. (2011). Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: proposal for a standardised pH-stat method. *Food Chemistry*, *126*(2), 498–505.

Lowe, M. E. (2002). The triglyceride lipases of the pancreas. *Journal of Lipid Research*, *43*(12), 2007–2016.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., & Mackie, A. (2011). The role of bile salts in digestion. *Advances in Colloid and Interface Science*, *165*(1), 36–46.

McClements, D. J. (2005). Theoretical analysis of factors affecting the formation and stability of multilayered colloidal dispersions. *Langmuir*, *21*(21), 9777–9785.

McClements, D. J., Decker, E. A., & Park, Y. (2008). Controlling lipid bioavailability through physicochemical and structural approaches. *Critical Reviews in Food Science and Nutrition*, *49*(1), 48–67.

Michalski, M. C., Genot, C., Gayet, C., Lopez, C., Fine, F., Joffre, F., … Committee, S. (2013). Progress in Lipid Research Multiscale structures of lipids in foods as

parameters affecting fatty acid bioavailability and lipid metabolism q. *Progress in Lipid Research*, *52*(4), 354–373. http://doi.org/10.1016/j.plipres.2013.04.004

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct. Food Funct*, *5*(5), 1113–1124.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T. O. R. S. T. E. N., Bourlieu, C., & Dufour, C. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food & Function*, *5*(6), 1113–1124.

Nakamura, A., Yoshida, R., Maeda, H., & M. Corredig. (2006). Soy soluble polysaccharide stabilization at oil–water interfaces. *Food Hydrocolloids*, *20*(2), 277–283.

Robinson, P. J., Smith, A. L., & Sly, P. D. (1990). Duodenal pH in cystic fibrosis and its relationship to fat malabsorption. *Digestive Diseases and Sciences*, *35*(10), 1299–1304.

Sarkar, A., Juan, J. M., Kolodziejczyk, E., Acquistapace, S., L., D.-C., & Wooster, T. J. (2015). Impact of protein gel porosity on the digestion of lipid emulsions. *Journal of Agricultural and Food Chemistry*, *63*(40), 8829–8837.

Schall, J., Bentley, T., & Stallings, V. (2006). Meal patterns, Dietary Fat Intake and pancreatic enzyme use in preadolescent children with Cystic Fibrosis. *Journal of Pediatric Gastroenterology and Nutrition*, *43*, 651–659.

Taylor, P., Mcclements, D. J., Decker, E. A., Park, Y., Clements, D. J. M. C., & Decker, E. A. (2009). Controlling Lipid Bioavailability through Physicochemical and Structural Approaches Controlling Lipid Bioavailability through Physicochemical, (December 2012), 37–41. http://doi.org/10.1080/10408390701764245

Turck, D., Braegger, C. P., Colombo, C., Declercq, D., Morton, A., Pancheva, R., & Schneider, S. M. (2016). ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clinical Nutrition*, *35*(3), 557–577.

Verger, R., & De Haas, G. H. (1976). Interfacial enzyme kinetics of lipolysis. *Annual Review of Biophysics and Bioengineering*, *5*(1), 77–117.

Woestenenk, J. W., van der Ent, C. K., & Houwen, R. H. (2015). Pancreatic enzyme replacement therapy and coefficient of fat absorption in children and adolescents with cystic fibrosis. *Journal of Pediatric Gastroenterology and Nutrition*, *61*(3), 355–360.

PAPER 7

LIPOLYSIS OF OIL AND BUTTER UNDER JOINT IN VITRO DIGESTION OF CARBOHYDRATE AND PROTEIN RICH FOOD MATRICES

Joaquim Calvo-Lerma [a,b*], Victoria Fornés-Ferrer[b], Ana Heredia[a] and Ana Andrés[a]

a Universitat Politècnica de València. Instituto de Ingeniería de Alimentos para el Desarrollo. Camino de Vera s/n. 46022 Valencia (Spain)

b Instituto de Investigación Sanitaria La Fe. Avenida Fernando Abril Martorell 106. 46026 Valencia (Spain). Tel. +34 961246712

* Corresponding author: joaquin_calvo@iislafe.es

ABSTRACT

The knowledge about the joint digestion of added fats and different food matrices is currently limited, given the complexity of the food system, gastrointestinal conditions and their interactions. This is particularly important in Cystic Fibrosis since patients are recommended to increase energy intake through the addition of oil or butter in their meals. Thereby the objective of the present work was to approach to the lipolysis of added fat (oil and butter) to different carbohydrate or protein rich matrices under in vitro digestion. Initial texture of the food samples, viscosity measurements of gastric digested medium and free fatty acids released after intestinal digestion were correlated. Linear mixed and beta regression models were applied to explain the results. Maximum force as texture parameter of the samples was directly correlated with viscosity of the gastric digested medium (p=0.01), and the latter to the total FFA released after intestinal digestion (p=0.048). Texture and nutrient composition of the matrices studied have an impact on viscosity conferred to the digestion medium and on total FFA release after the intestinal digestion.

KEYWORDS: in vitro digestion, food matrix, lipolysis, free fatty acids, texture, viscosity

1. INTRODUCTION

In the recent years, the study of lipid digestion in terms of bioaccessibility and bioavailability has gained importance in the nutrition and food science research fields, for the direct implication of their overconsumption in the development of non-communicable diseases such as type II diabetes and obesity [2,3]. In contrast, there are other diseases in which the study of lipolysis is of upmost importance, the goal being trying to achieve the maximum lipolysis.

This is the case of cystic fibrosis, in which pancreatic insufficiency impairs digestive enzymes release to the small intestine, impeding the digestion of nutrients, especially fat [4,5]. The lack of pancreatic secretion also implies a lower pH at this section of the digestion tract [6,7]. In addition, bile salts secretion is also altered in the CF scenario, with an up to 10 times lower concentration [8]. To palliate this disorder, pancreatic enzyme replacement therapy (PERT) consists of the exogenous administration of enzymes in every meal [9]. However, a scientifically valid dosing criterion is inexistent, and several authors have claimed for the study of foods as possible determinants of the process of digestion with the enzymatic supplements [10]. In fact, this situation was addressed by MyCyFAPP project in order to study food properties by means of an in vitro digestion model as the key element to adjust PERT [11]. Previous research of this project evidenced that increasing doses do not always mean increased lipolysis [12]. In another study we concluded that, while under standard in vitro digestion conditions (healthy adult), the lipid structure within the food matrix had no effect on lipolysis, under the CF simulated conditions this had a significant impact [13].

The food matrix can be defined as the spatial architecture resulting from the assembly of macromolecules such as proteins, carbohydrates and lipids into a coordinated network. It plays a crucial role in how food interacts with the gastrointestinal tract and the resulting release and uptake of nutrients [1]. Lipids in foods are present in a wide variety of forms, either in processed foods as ingredients or in naturally-occurring foods within a food matrix. Additionally, many food recipes include the addition of oil (i.e. salads) or butter (i.e. fried fish).

In the recent years, in vitro digestion methods have allowed for the study of several aspects related to lipolysis, such as the triglyceride composition, the stereospecific position of the fatty acids in the triglyceride molecule or the type of emulsifiers [14,15]. However most of this research has been conducted on the basis of model foods or emulsions, thus limiting the generated knowledge to the macromolecular scale. On the other hand, few studies have addressed the complexity of the real food matrix at a macroscopic level [1]. It has been recently demonstrated that, besides these factors affecting lipolysis, the nutrient interaction does also play an important role on fat digestion fate [13]. Overall, shading light on the factors affecting lipolysis in real foods is a real challenge, given the concurrency of several parameters at different levels of complexity. What is more, the fate of nutrient digestion when different foods are consumed together could lead to an even more complex scenario, which to our knowledge has been never addressed.

Thus, in order to study the influence of the food matrix on lipolysis of added fat, the objective of the present work was to assess the lipolysis of oil and butter under joint in vitro digestion of carbohydrate and protein rich food matrices.

2. METHODS

2.1. Materials

Five fat-free food matrices (<1% fat) were selected (**Table 4.15**): one rich in protein and carbohydrates (bread), one rich in carbohydrates (potato), and three rich in protein of different types: casein (degreased cheese) and fibrilar proteins (hake and turkey). To these matrices, either olive oil or butter was added. The selection of the added fats was made following these criterion: one animal-origin fat with a saturated fatty acids profile (butter) and one vegetal-fat with an unsaturated fatty acids profile (olive oil). Before texture measurement and in vitro digestion hake and potato were cooked with a microwave (600W, 3 min) while bread, fresh cheese and turkey were used in their raw form.

Table 4.15. Characterisation of the foods assessed: type of food matrix and nutrient composition

		Type of matrix	Protein (g/100g)	Carbohydrates (g/100g)	Lipids (g/100g)	FFA profile
Fat-free food matrices	Bread	Carbohydrates (network)	9.6	45	1	-
	Potato	Carbohydrates (granules)	2.3	14.8	0.1	-
	Fresh cheese	Protein (network)	7.7	0	0.1	-
	Turkey	Protein (fibres)	17.8	0.6	0.5	-
	Hake	Protein (fibres)	15.8	0	1	-
Added fat	Olive oil	Bulk fat	0.1	0	99.8	Mono-unsaturated
	Butter	Water-in-oil emulsion	0.8	0.8	81	Saturated

Pancreatic enzyme supplements (Kreon® 10000 LU) were kindly donated by Hospital Universitari i Politècnic La Fe (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme in the shape of gastro-resistant microspheres equivalent to 10000 lipase U, 8000 amylase U, and 600 protease U.

For the preparation of the simulated digestive fluids, the following chemicals were needed: pepsin from porcine gastric mucosa (≥2500 U / g protein), bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$ and $CaCl_2$ all of them from

Sigma-Aldrich Chemical Company (St Louis, MO, USA). NaOH (1 N) and HCl (1 N), were acquired from *AppliChem Panreac*. For the GC-MS analytical determinations, Triton-X 100 %, trichloroacetic acid (TCA) and hexane were required, as well as the analytical standards capric acid, lauric acid, palmitic acid, mystiric acid, stearic acid, oleic acid, linoleic acid, alpha – linoleic acid and arachidic acid. All of them were provided from Sigma-Aldrich Chemical Company (St Louis, MO, USA).

2.2. Experimental design

The five fat-free food matrices were individually digested with both olive oil and butter in order to characterise the effect of the type of fat on digestion when combined with different structures, and then combinations of two and three matrices were digested with olive oil in order to assess the impact of the double and triple interactions occurring among components (meal factors). In all the experimental sets the proportion matrix/added fat was 4.5g matrix/0.5g fat: in the case of 2 matrices the amount of each was thus 2.25g and when examining 3 matrices the amount of each was 1.5g. **Table 4.16** shows the experimental design.

All the samples were in vitro digested mimicking the intestinal conditions of cystic fibrosis patients (pH 6), bile concentration 1mM) and using the recommended pancreatic enzyme supplement dose of 2000 LU/g fat. According to previous research (REF) food properties did not show a significant effect on lipolysis extent when simulating healthy digestion conditions, i.e. intestinal pH7 and bile concentration 10 mM, thus they were not included in the design. All the experiments were conducted in triplicate.

Table 4.16. Experimental design. Combination of the naturally fat-free food matrices with the type of added fat.

	Bread	Potato	Cheese	Turkey	Hake
No interaction					
Double interaction					
Triple interaction					

Added Olive oil Added Butter

2.3. In vitro digestion process

Food samples (5g in total) were placed into 50 mL falcon tubes. Each tube was then used for a simulated gastrointestinal in vitro digestion process. The methodology applied for the present study was based on the international procedure consisting in three different stages, oral, gastric and intestinal, established by the cost action-INFOGEST and published by Minekus et al., (2014). The digestion fluids were prepared fresh daily from stock solutions (**Annex 3**). The enzymatic activity was tested before each experiment following the protocol proposed by Carrière et al., (2000).

Oral stage: Simulated salivary fluid (5 mL) (SSF; pH 8) at 37 ºC, was added to the food sample in a ratio 1:1 (v/w) and properly homogenized with a kitchen blender for 3 minutes (Vario Mixer, Ufesa 600 W).

Gastric stage: After the oral stage, simulated gastric fluid (SGF; pH 3) was added to each tube containing the oral bolus (1:1 v/w). Pepsin was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). The pH of the mixtures was adjusted with HCl (1N) to pH 2.8 ± 0.1 and samples were rotated head- over-heels at 55 rpm for 2 h at 37 ºC using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and an incubator chamber Selecta (JP Selecta SA, Barcelona). These mixing conditions provided constant mechanical energy to induce the breakdown of the food matrix during digestion.

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF; pH 6 7) containing the enzymes (1000-4000 lipase units (LU)/g of fat), was added in a proportion 1:1 (v/w) to each tube containing the gastric chime. The pH of the mixtures was adjusted to pH 6.0 ± 0.1 or 7 ± 0.1, depending on the experimental design, with NaOH (1N). Samples were then rotated head-over-heels at 55 rpm for another 2 h at 37 ºC. pH was monitored during the digestion process and readjusted if necessary as pH below 5.7 might inactivate lipase activity (29,30). After 2 hours of intestinal stage samples were placed in ice and pH adjusted to 9 to ionize free fatty acids and stop lipase activity.

2.4. Texture measurements

Textural properties were evaluated by means of a double compression test using a tesxture analyser (mod. TA-XT PlusAname, Spain) equipped with a 50 kg load cell. Texture test was performed on the five matrices: bread, turkey and fresh cheese were raw and potato and hake were cooked. Samples were cut to a cubic form with 1cm. TPA parameters were measured at room temperature according to the following procedure: probe contact area 113 mm^2, crossbar speed 5mm s^{-1}, final strain 50%, surface sensing force 0.049N, force threshold 0.098N and time between first and second stroke 1 s.

The textural measurements of raw and cooked meat were made using a TA-XT2 texture analyser with XT- RA dimention software. Meat samples were cut by cork-bore

to a cylindrical form with 6 mm radius and approximately 13 mm in height. TPA parameters were measured at room temperature, according to the following testing procedure: probe contact area 113 mm2, crossbar speed 5 mm s^{-1}, ®nal strain 50%, surface sensing force 0.049N, force threshold 0.098N, time interval between ®rst and second stroke 1 s.

2.5. Viscosity measurements

Viscosity of the digesta before (Gt_0) and after (Gt_1) the gastric stage was determined by the Bostwick consistometer method. It measures the distance (d) the fluid displaces in a steeped surface in a scale ranging from 0 to 26 cm, so that the higher the viscosity the lower the distance displaced (d^{-1}).

2.6. Free fatty acid measurement

The micellar phase (drained digested samples after intestinal digestion) was freeze-dried overnight. Then samples were submitted to a transesterification to methyl esters (FAMEs) with BF3 and methanol at 20 °C according to the IUPAC standard method (IUPAC, 1992: Yaich et a., 2011). The fat extraction was effectuated by adding 3 ml of hexane in 15 ml falcon tubes and by rotating head-over-heels at 55 rpm during 90 minutes using Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia). Then tubes were centrifuged for 5 minutes 1000 rpm and 1 ml of supernatant was dried with nitrogen flow and the residue was used for methylation. After it, 50 µl of internal standard (pentatioic (1 mg · ml-1), 40 µl of Hexane and 100 µl of BF3 were added in the vial with the residue obtained, vortexed 15 seconds and heated at 70 °C during 90 minutes. Then 100 µl of NaCl (25 % w/v), 40 µl of H2SO4 (10 % w/v) and 700 µl of Hexane were put in the mix, vortexed 15 seconds and settled for 30 minutes. After that time 700 µl of upper layer was taken and it was transferred to the injection vial ready to analyse. Pentanoic acid was used as internal standard.

Samples were analysed with an Agilent 5977A system and an HP – 5 MS UI (agilent) (30 m x 0.25 mm, 0.25 µm film thickness) was used with helium. The oven

temperature was programmed at 90 °C for 2min, increased to 222 °C at 5 °C/min for 5 min, and increased to 280 °C at 20 °C/min for 2 min; split flow was adjusted at 1mL/min, and injector temperature was at 280 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 30 to 650. Identification of components was carried out by comparison of their matching against commercial (Nis 11t, Nist_msms, mainlib, replib, wiley7n) libraries, built by genuine compounds and components, as well as MS literature date, was also used for the identification

The software used for data acquisition and processing was 6890. Data analysis identification and quantification of FAMEs was accomplished by comparing the retention times of the peaks with those of pure standards (Supelco®37 Component FAMEs Mix, Sigma), and analysed under the same conditions.

2.7. Statistical analysis

Data were summarised using mean, standard deviation, median and 1st and 3rd quartile in the case of continuous variables and with absolute ant relative frequencies in the case of categorical variables.

To study the effect of Hardness on Viscosity, a linear regression model was carried out. In addition, a mixed linear regression model was performed to assess the association between the total FFA and the predictive variables protein, fat, carbohydrates and viscosity, all of them transformed logarithmically to guarantee a normal distribution. A random effect was included in the model to correct the effect of the food combination. Eventually, the effect of the protein and carbohydrates' hardness on the viscosity was assessed using a linear regression model. The interaction between protein hardness and carbohydrates hardness was considered to check the effect of the combination.

The analyses were carried out using R software (version 3.5.0) and packages clickR (version 0.3.64), lme4 (version 1.1-17) and lmerTest (version 3.0-1). P-values below 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Food properties, viscosity of gastric digestion media and lipolysis

Textural parameters of the food matrices were assessed in order to characterise their structural properties (**Table 4.17**). Turkey presented the highest hardness (55.1 N) and bread the lowest (2.3 N), potato, fresh cheese and hake having similar intermediate values. Potato presented the highest adhesiveness (-63.25 g · sec) as compared to the other samples, followed by turkey (-17.6 g · sec), the rest of the foods presenting a similar low result of this parameter. Potato showed low cohesiveness (0.11) as compared to the other matrices, which ranged 0.58-0.75. The hardness was selected as the most representative and reliable property of the 5 foods given their different natures. It is well known that the nutrition composition of the foods determines their physical characteristics (REFS). In the study foods, the main nutrient was either carbohydrates or protein (**Table 4.16a**). Therefore, in potato and bread carbohydrates were the only responsible for the hardness these samples presented, while protein was the responsible of this parameter in fresh cheese, turkey and hake.

Table 4.17. Textural properties of the naturally fat-free food matrices as measured by a TPA double compression assay. Values expressed as mean (SD)

Food	Hardness (N)	Adhesiveness (g · sec)	Cohesiveness	Chewiness
Bread	2.35 (0.5)	-2.24 (0.41)	0.75 (0.04)	1.65 (0.34)
Potato	10.97 (4.3)	-63.25 (33.88)	0.11 (0.03)	0.37 (0.22)
Fresh cheese	6 (0.89)	-4.51 (0.92)	0.78 (0.04)	4.68 (0.82)
Turkey	55.11 (6.52)	-17.6 (16.95)	0.65 (0.05)	32.61 (7.35)
Hake	8.41 (2.51)	-6.84 (4.5)	0.58 (0.06)	3.61 (1.34)

When aiming at elucidating the physical food properties affecting lipolysis, several authors have found associations between the presence of some nutrients and the viscosity of the digestion medium. Lamothe et al. (2012) concluded that in cheese, the high textural properties were associated to a slower degradation in the gastric phase [16]. Then, these cheese matrix properties also influenced the lipase access to lipids in the intestinal stage, since the higher the viscosity prevented the diffusion of the enzymes [3].

In our sample, the hardness of the sample was associated to the viscosity conferred to the digestion medium (p=0.01) so that the higher the hardness the lower the viscosity (d^{-1}). In addition, we studied the impact of nutrient composition and the viscosity conferred to the gastric digestion medium on total FFA release (**Table 4.18**), finding a significant decreasing effect of protein (p=0.025) and a significant decreasing effect of viscosity (d^{-1}) (p=0.048).

Table 4.18. Influence of carbohydrates and protein content, hardness and viscosity of the gastric digestion medium on total FFA released after intestinal digestion.

	Estimate	Lower 95%	Upper 95%	P-value
Hardness	4.36	1.35	7.36	0.01
Protein	-0.783	-1.391	-0.175	0.025
Carbohydrates	0.229	-0.1	0.558	0.21
Viscosity (Gt_0)	1.185	0.133	2.236	0.048

Thus, both food matrix properties and the viscosity these confer to the digestion medium have an effect on total FFA release.

3.2. Interactions between different foods and viscosity

As explained above, food matrix determines the viscosity of the digestion medium during the gastric stage. Moreover, **Figure 4.17** represents the additive effect

of the food matrix properties when different foods where combined on viscosity. In this sense, for example, turkey presents high hardness and low viscosity (high d), and bread has low hardness and high viscosity (low d). When combining these two foods together in the gastric digestion, the sum of the textural properties result in a proportional sum of the viscosities conferred.

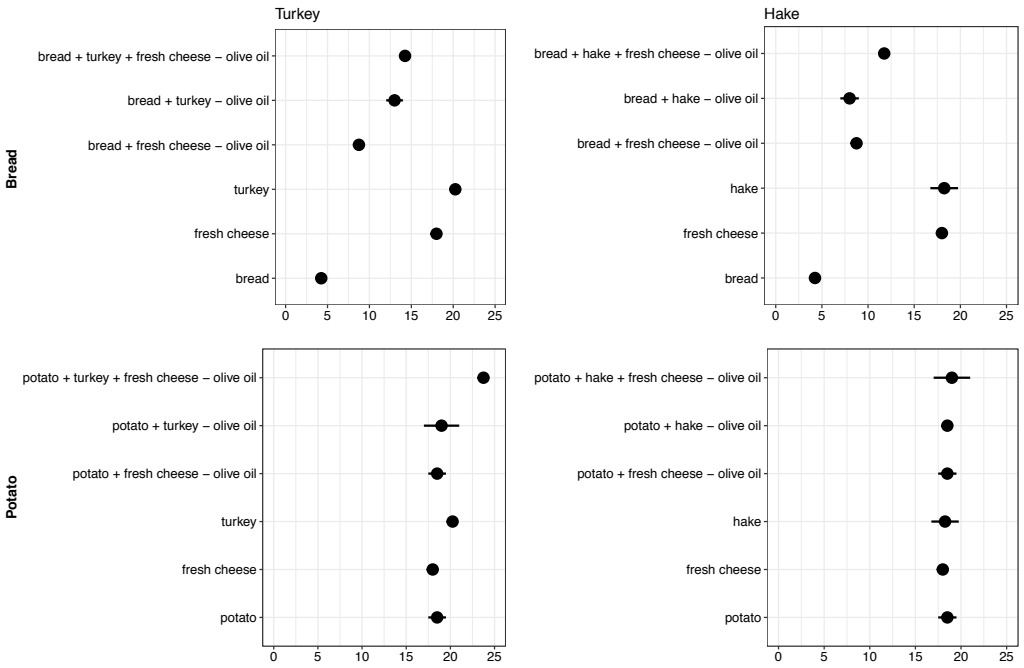


Figure 4.17. Viscosity conferred to the digestion media by the food matrices individually and when combined with each other. Viscosity is expressed in distance covered by the digestion fluid.

3.3. Interactions between foods and FFA release

In the present section a description of the induvial FFA release and the overall FFA profile is presented, including the food matrices digested in isolation with olive oil or butter, the combination of a CH and a protein matrix and the combination of a CH and two protein matrices.

3.3.1. Individual food matrices with olive oil or butter

The effect of the five food matrices properties caused differences in the amount of FFA released from the digestion of olive oil and butter (**Figure 4.18**). Overall, in butter the amount of FFA was lower than in olive oil, but in both cases, potato was the matrix allowing for the highest release of the individual FFAs, and bread for the lowest. The addition of butter to the food matrices led to the same FFA profile in all of them: C16:0 was found in the highest amount (≅ 7.5g), followed by C18:0 and C18:1, which were found in similar amounts (≅ 3g). The rest of the assessed FFA were found in very low amounts. Potato was the food that allowed for the highest release of all the assessed FFA, and fresh cheese for the lowest. Bread and turkey presented a similar amount of FFA, especially for C16:0 and C18:0 and hake led to an in-between result. In the case of olive oil, the FFA profile was characterised by C18:1 in high amounts, followed by C16:0 and C18:0 and C18:2 in lower amounts; the presence of the other FFAs was not detected. In this scenario, potato was also the food leading to the highest release of all the FFA. Fresh cheese and turkey were those matrices leading to the lowest FFA release, while hake and bread presented an intermediate amount.

3.3.2. Combination of a Carbohydrate and a protein matrix

In contrast, when combining a CH matrix with a protein matrix, the big difference in the amount of FFA released in the contexts of digestion of olive oil with only potato and bread was minimised. Both CH-matrices showed a very similar FFA profile (**Figure 4.19**), although in bread, amounts released were higher. This profile was characterised by high release of C18:1, followed by C16:0 and C18:0 and then C18:2. In bread, fresh cheese and turkey presented the same amount of FFA and hake led to lower amounts. In potato, fresh cheese and hake had the same profile and turkey allowed for a higher release of FFA. Noteworthy in bread a small presence of C10:0, C12:0 and C14:0 (<1mg/100g product) was found, but not in potato, this possibly being related to the small amount of fat naturally present in bread but not in potato.

3.3.3. Combination of a Carbohydrate and two protein matrices

Finally, the combined digestion of olive oil with one CH matrix plus two protein matrices, fresh cheese plus turkey or hake, the FFA profile was similar as in the previous experiment. As shown in **Figure 4.20**, in this scenario the presence of turkey or hake did not lead to differences in the profile, and the only conditioning factor on the amount of total FFA was the type of CH matrix, i.e. digestions with bread led to slightly higher FFA release.

4. CONCLUSION

In conclusion, texture and nutrient composition of the matrices studied have an impact on viscosity conferred to the digestion medium and on total FFA release after the intestinal digestion. Besides, texture influences the viscosity, and this association is maintained even when different matrices from different nature are combined. Finally, the food matrix influences the amount of individual FFA released.

ACKNOWLEDGEMENTS

Authors of this paper acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme (PHC-26-2014 call Self-management of health and disease: citizen engagement and mHealth) for fully funding this research under grant agreement number 643806.

REFERENCES

1. Guo, Q., Ye, A., Bellissimo, N., Singh, H., & Rousseau, D. (2017). Modulating fat digestion through food structure design. *Progress in lipid research*, *68*, 109-118.
2. G. Bray, B. Popkin, Dietary fat intake does affect obesity, Am. J. Clin. Nutr. 68(6) (1998) 1157-1173.
3. E. Jéquier, Pathways to obesity, Int. J. Obes. 26(Supplement 2) (2002) S12-S17.

4. O'Sullivan, B.P., Freedman, S.D. (2009) Cystic fibrosis. *Lancet*, 373:1891– 904.
5. Sikkens, E. C., Cahen, D. L., Kuipers, E. J., & Bruno, M. J. (2010). Pancreatic enzyme replacement therapy in chronic pancreatitis. *Best Practice & Research Clinical Gastroenterology*, *24*(3), 337-347.
6. Gelfond D, Ma C, Semler J, Borowitz D (2013) Intestinal ph and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. Dig Dis Sci 58:2275–81.
7. Robinson PJ, Smith AL, Sly PD, Al RET (1990) Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. Dig Dis Sci 35:1299–304.
8. Norman AP. (1979) Intestinal bile salts in cystic fibrosis. Archives of disease in childhood 54:19-24
9. Fieker, A., Philpott, J., & Armand, M. (2011). Enzyme replacement therapy for pancreatic insufficiency: present and future. *Clinical and experimental gastroenterology*, *4*, 55.
10. Turck, D., Braegger, C. P., Colombo, C., Declercq, D., Morton, A., Pancheva, R., ... & Schneider, S. M. (2016). ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clinical Nutrition*, *35*(3), 557-577.
11. Calvo-lerma, J. et al. (2017) Innovative approach for self-management and social welfare of children with cystic fibrosis in Europe : development, validation and implementation of an mHealth tool (MyCyFAPP). Br. Med. J. Open 7:e014931
12. Calvo-lerma, J. et al. (2018) Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 1, in vitro study. Journal of Cystic Fibrosis (under review)
13. Calvo-lerma, J. et al. (2018) In vitro digestion of lipids in real foods: influence of lipid structure within the food matrix and interactions with non-lipid components. Journal of food Science (under review)

14. Li Y, McClements J (2010) New Mathematical Model for Interpreting pH-Stat Digestion Profiles : Impact of Lipid Droplet Characteristics on in Vitro
15. Digestibility. J Agric Food Chem 58:8085–92.
16. Ozturk B, Argin S, Ozilgen M, Mcclements DJ (2015) Nanoemulsion Delivery Systems for Oil-Soluble Vitamins : Influence of Carrier Oil Type on Lipid Digestion and Vitamin D 3 Bioaccessibility. Food Chem 187:499-506
17. Lamothe, S., Corbeil, M. M., Turgeon, S. L., & Britten, M. (2012). Influence of cheese matrix on lipid digestion in a simulated gastro-intestinal environment. Food & function, 3(7), 724-731.

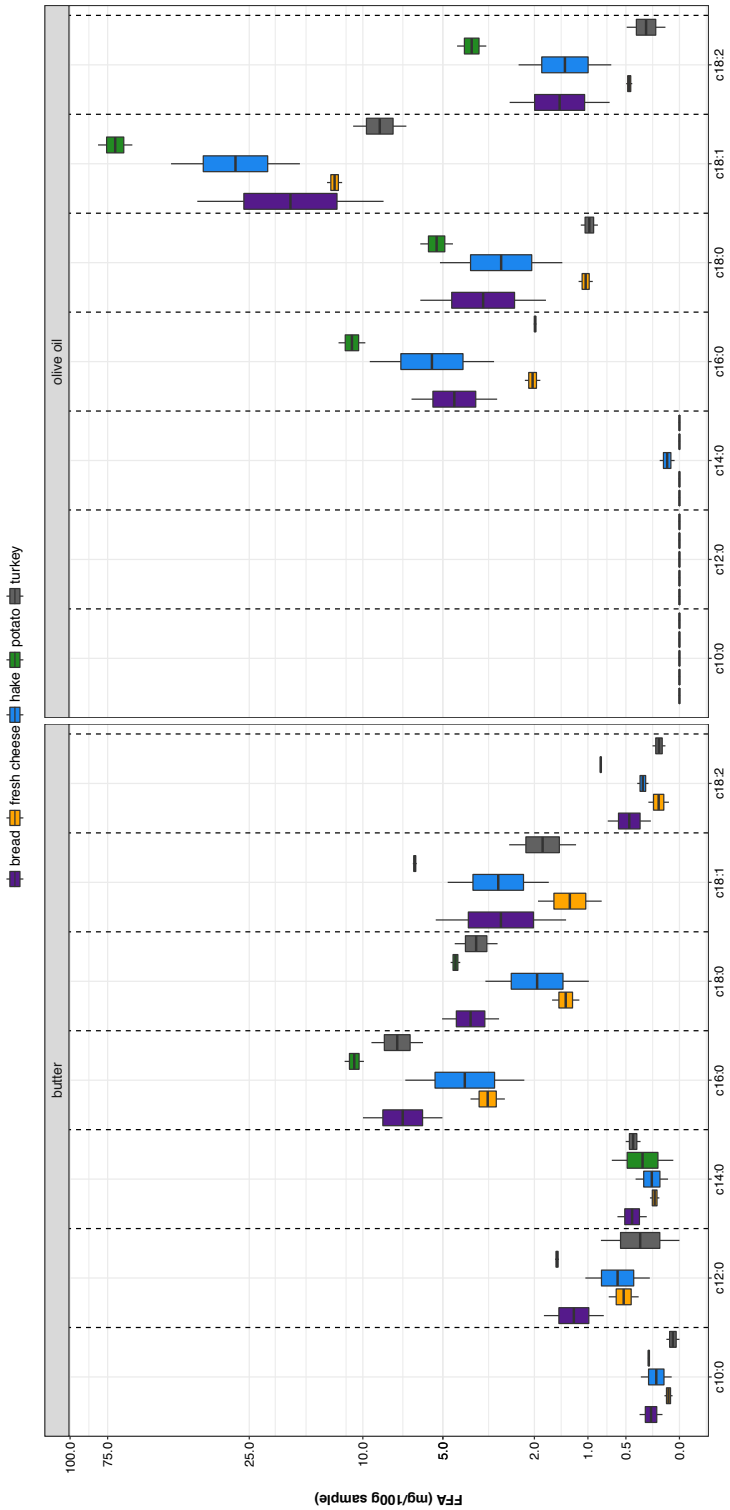


Figure 4.18. Free fatty acids profile of butter and olive oil when digested with bread, fresh cheese, hake, potato and turkey.

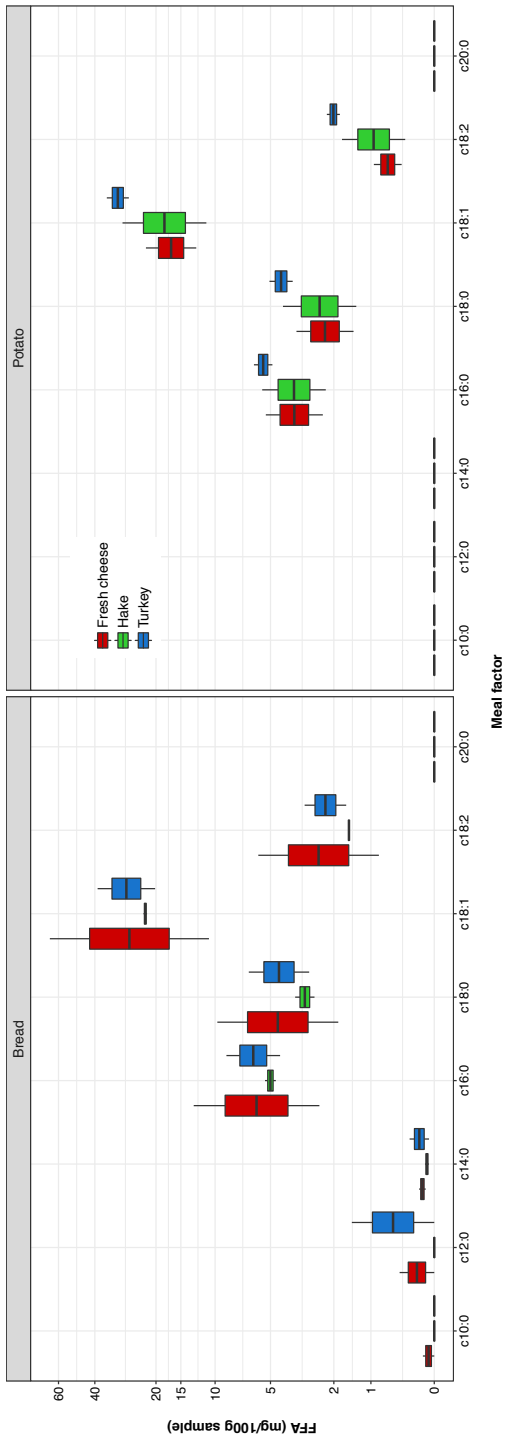


Figure 4.19. Free fatty acids profile of olive oil when digested with a carbohydrate matrix (bread or potato) and a protein matrix (fresh cheese, hake or turkey)

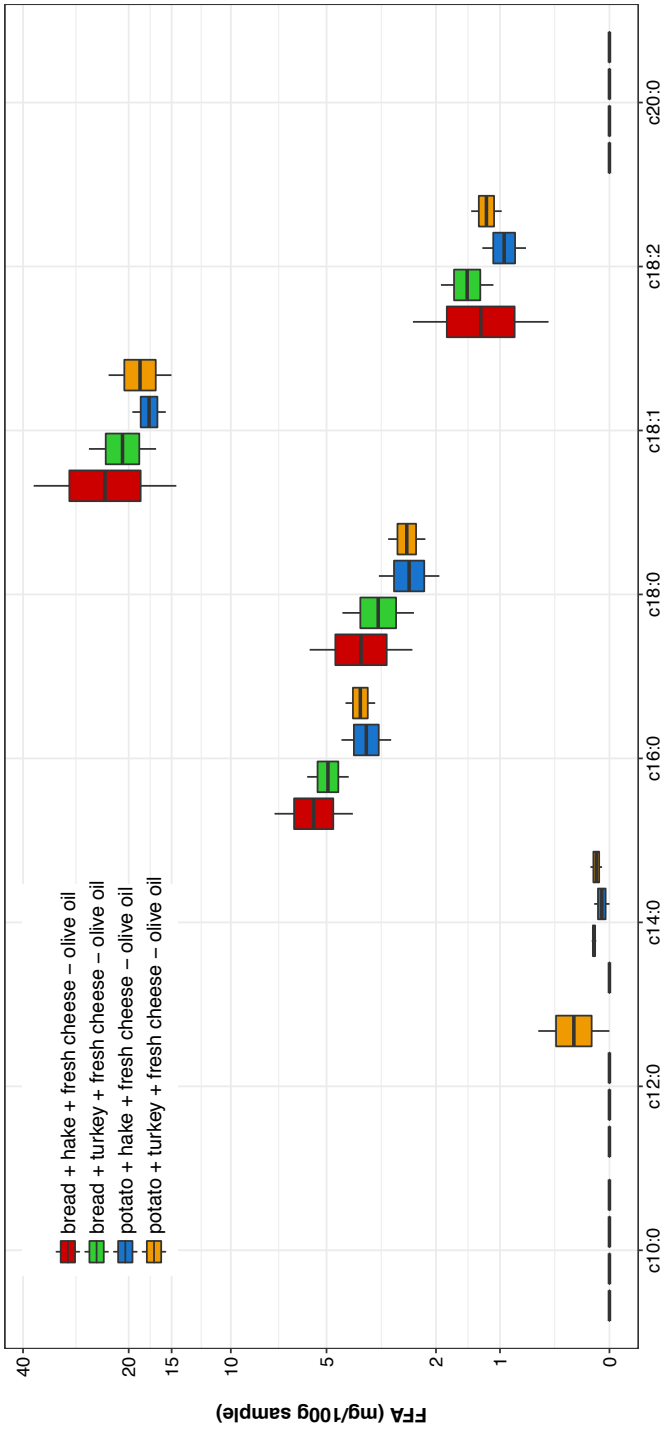


Figure 4.20. Free fatty acids profile of olive oil when digested with a carbohydrate matrix (bread or potato) and two protein matrices (fresh cheese and hake or turkey)

CHAPTER 4

AN EVIDENCE-BASED METHOD TO ADJUST PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS

PAPER 8

Calvo-lerma, J. et al. (2018) *Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 1, in vitro study*. Journal of Cystic Fibrosis

PAPER 9

Calvo-lerma, J. et al. (2018) *Evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis: Part 2, in vivo validation of the in vitro model*. Journal of Cystic Fibrosis, (Under review)

PAPER 10

Calvo-lerma, J. et al. (2018) *Association between faecal pH and coefficient of fat absorption in children with cystic fibrosis on a controlled diet and dose of pancreatic enzyme replacement therapy.* Journal of Cystic Fibrosis (Under preparation)

PAPER 8

EVIDENCE-BASED METHOD TO ADJUST PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS: PART I, IN-VITRO STUDY

Joaquim Calvo-Lerma[1,2*], Victoria Fornés-Ferrer[2], Irene Peinado[1], Ana Heredia[1], Carmen Ribes-Koninckx[2], and Ana Andrés[1*] on behalf of MyCyFAPP

[1]Universitat Politècnica de València, Research Institute of Food Engineering for Development, P.O. Box: 46022, Valencia, Spain. Tel (+34) 963879412

[2]Instituto de Investigación Sanitaria La Fe de Valencia, P.O. Box 46026, Valencia, Spain. Tel (+34) 961246712

* Corresponding author

ABSTRACT

Objectives: An evidence-based method to adjust Pancreatic Enzyme Replacement Therapy (PERT) is inexistent, and lipid content of meals is used as a rough criterion. In this study, an *in vitro* digestion model was set up to determine the theoretical optimal dose (TOD) of PERT for a selection of foods.

Methods: Eight meals were *in vitro* digested under CF gastrointestinal simulated conditions (intestinal pH6, bile 1mM) with a range of PERT doses. Results in terms of % of lipolysis extent were fitted into a linear-mixed segmented model and the deducted equations were used to predict the TOD to reach 90% of lipolysis in every food. In addition, the effect of intestinal pH and bile concentration were investigated.

Results: The predictive equations obtained were characterized by a change point in the lipolysis extent when increasing PERT dose. From this change point, the lipolysis extent could increase with dose (as for milk) or decrease (pizza). This means that for the assessed foods, lipolysis was not only dependent on PERT dose and their lipid content. Moreover, intestinal pH and bile concentration had significant effects on lipolysis.

Conclusion: Foods' response to lipolysis with PERT has been tested for the first time under CF simulated gastrointestinal conditions. The results evidenced that depending

on food characteristics, a specific TOD should be assigned to achieve an optimal digestion extent. Thus, this work represents a first step towards an evidence-based method for PERT dosing, which will be applied in an *in vivo* setting to validate its efficacy.

Keywords: cystic fibrosis*; in vitro digestion; exocrine pancreatic insufficiency; enzymes; lipid digestion; linear mixed segmented model*

1. INTRODUCTION

Exocrine pancreatic insufficiency (PI) is an associated disorder to Cystic Fibrosis (CF) affecting 85-90 % of the patients [1]. The obstruction of the pancreatic duct in CF leads to a decrease in the secretion of sodium bicarbonate and pancreatic juice containing digestive enzymes to the intestine, leading to nutrients' maldigestion and malabsorption [2,3] [4].

Currently, Pancreatic Enzyme Replacement Therapy (PERT) consists of the exogenous administration of a porcine-origin enzyme supplement to promote nutrients' digestion and absorption [5]. The implementation of PERT in the regular treatment of the CF led to a great improvement of nutrients' digestion and absorption. However, it has been shown that patients are not able to achieve and maintain satisfactory levels of fat absorption over the years [6,7]. Clinical trials aimed at elucidating maldigestion in CF have led to inconsistent conclusions [6], and despite the fact that several authors have highlighted the need of generating knowledge to establish an accurate method, an evidence-based method for PERT dosage remains inexistent[8,9]. Therefore, lipid content of meals and/or patients' body weight are currently the only available parameters to roughly guide health professionals and patients to adjust PERT doses. The new European Guidelines in CF council a range of 2000-4000 lipase units per gram of lipids (LU/ g lipid), although acknowledging a low degree of evidence [10].

Moreover, a recent study has elucidated that there are huge differences in the dosing of PERT among European countries, showing large variability within and between patients, without association with weight or BMI [11]. This pattern of intake compromises the recommendations of adjusting the enzymes' dose to the lipid content of the meals, since this lipid content largely varies from one meal to another and from one day to another [11].

Furthermore, intrinsic food factors, have been pointed out as determinants in the process of gastrointestinal nutrient digestion and thus in the PERT effectiveness [5,8,12–16]. Food matrix physicochemical properties or lipids origin have proved to highly influence the process of lipids hydrolysis [17,18]. Likewise, the gastrointestinal environmental conditions have demonstrated to play a key role on lipids digestion [8,19–21]. In the particular case of CF, specific differences as compared to the healthy population take place in the intestine (pH and bile concentration) due to the pancreatic insufficiency and altered biliary function [18,19,22].

In this context, *in vitro* digestion studies of different foods might suppose a useful tool to shed light on the understanding of lipolysis in CF-specific conditions and to give guidance on PERT dosing. They allow for the controlled, accurate and reproducible in-lab simulation of the physiological gastrointestinal conditions [23]. Nevertheless, up to now, there are only a few known investigations about lipid digestion in real or complex foods, what limits the translation of knowledge from *in vitro* digestion outcomes to clinical practice [24,25].

In the above-described scenario, MyCyFAPP Project (www.mycyfapp.eu) pursues CF patients' self-management of PERT supported by a mobile application by means of a new dosing predictive model [26]. Within the frame of MyCyFAPP, the aim of this study is setting up an evidence-based method for PERT adjustment by applying a static *in vitro* digestion method in order to obtain the theoretical optimal dose of PERT (TOD) for a selection of foods.

2. METHODS

2.1. Materials and test foods

Pancreatic enzyme supplements (Kreon® 10,000 LU), were kindly donated by "Hospital Universitari Politècnic La Fe" (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme in the shape of gastro-resistant microspheres equivalent to 10,000 lipase U, 8,000 amylase U, and 600 protease U. The other chemicals used for the *in vitro* digestion were: pepsin from porcine gastric mucosa (3200-4500 U/mg), bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$ y $CaCl_2$ and Triton X-100 obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA), Ethanol obtained from Vidrafoc (Barcelona, Spain) and NaOH, 1 N and HCl 1 N acquired from *AppliChem Panreac*. Food products were purchased at a local supermarket.

A total of eight foods covering a wide range of lipid contents were selected: salad with olive oil (9.4% lipids), pizza (7.4%), Greek-style yoghurt (10%), ham and cheese sandwich (7.9%), milk (3.6%), buttery bread (24%), breakfast cereal (4%) and chocolate biscuits (27%). The selection was made upon some of the most consumed food products identified in a paediatric CF population (N-P-037, ESPGHAN 50th Annual Meeting). It included different food structures (i.e. emulsions, fibres, proteinaceous, etc.), with different lipid types (animal and vegetal origin).

2.2. Study design

Two sets of experiments were conducted for each of the tested food products. The first one was aimed at elucidating the influence of the gastrointestinal factors on the lipolysis extent. In this set, the dose of PERT was fixed at 2000 LU/g fat and intestinal pH and bile concentration were the variables with two levels each: pH 6 and pH 7, and bile concentration 1 mM and 10 mM, giving four pH/bile combinations: 6/1, 6/10, 7/1 and 7/10.

The second set of experiments was established to assess PERT dose under fixed GI conditions. These conditions were selected as the worst possible scenario in the small intestine digestion in CF patients, namely: the altered biliary function leads to a

lower bile concentration in the intestine [27,28], and the obstruction of the pancreatic duct to a lower pH as a consequence of the lower secretion of sodium bicarbonate [2,3,22,29,30]. So, intestinal pH and bile concentrations were fixed at 6 and 1mM respectively and the variable was PERT dose, with five levels: 0, 1000, 2000, 3000 and 4000 LU/ g fat. Results from this experimental set were modelled to obtain predictive equations for TOD calculation.

All the experimental conditions and analyses were conducted at least in triplicate.

2.3. *In vitro* digestion simulation

The digestion process was simulated according to the static standardised method proposed by Minekus et al., (2014) [23] which establishes the "smallest common denominator" of the standard conditions that are close to the physiology of a healthy adult, and thereafter amendments were applied according to the scope of this research [23]. The static digestion process was simulated in three stages.

Oral stage: The food samples (5 g), were mixed in a proportion 1:1 (w/v) with simulated salivary fluid (SSF), and properly homogenized during 2 minutes. The mixture was then placed in 50 mL falcon tubes and incubated for 5 min at 37 ºC.

Gastric stage: Then, simulated gastric fluid (SGF) (pH 3) was added in a proportion 1:1 (w/v) to each tube containing the oral bolus. The pH of the mixtures was readjusted to pH 3 with HCl (1N). Pepsin was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). At this point, PERT was added into the tubes in a concentration established in the experimental design. Samples were rotated head-over-heels at 55 rpm for 2 h at 37 ºC using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and an incubator chamber Selecta (JP Selecta SA, Barcelona). These mixing conditions provided constant mechanical energy to induce the breakdown of the food matrixes during digestion, as occurs in the physiological process.

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF) (pH 6), was added in a proportion 1:1(w/v) to each tube containing the gastric chyme. Bile

was added to the SIF in order to reach a final concentration in the intestinal mix of either 1mM or 10 mM depending on the experimental design. The pH of the mixtures was adjusted, with NaOH (1N), to pH 6 or 7, depending on the experimental design. The samples were then rotated head-over-heels at 55 rpm for other 2 h at 37 ºC. pH was monitored during the digestion process and readjusted if necessary as pH below 5.7 might inactivate lipase activity [23,24]. After two hours of digestion, samples were placed in an ice bath for ten minutes and pH was adjusted to 9 to stop enzymatic activity [31,32].

Fluids' composition required for each digestion stage, were those described by Minekus et al., 2014 [23] (**Annex 3**); they were prepared fresh daily and kept at 37 ºC before their use.

2.5. Lipolysis analysis

Lipolysis extent was determined by measuring the free fatty acids (FFA) released from initial triglycerides content of foods after the complete simulated gastro intestinal digestion. The liquid phase from the digested samples (100 μl) was separated by sieving (1 mm^2) and mixed with 10 mL of a solution made of 5.6 % Triton X-100 and 6 % ethanol in water, to solubilize the free fatty acids and ensure to stop lipase activity. The free fatty acids were measured on the diluted samples using a free fatty acid spectrophotometric assay kit (Roche Diagnostics, Indianapolis, IN, USA) in a spectrophotometer (UV/vis, *Beckman Coulter*) [33]. Palmitic acid standard was used for quantitative determination of FFA. Digested fat was estimated assuming the release of 2 moles of fatty acids per 1 mole of triglycerides [33]. Values higher than 100% are inherent to the internal error of the analytical method used to quantify FFA.

2.6. Statistical analyses

Experimental data were summarised by mean (standard deviation) in case of continuous variables (PERT dose) and by relative and absolute frequencies in case of categorical variables (pH, bile concentration). The association studies between lipolysis

and extrinsic factors were analysed by linear mixed regression considering each different food as a random factor. These models were fitted for the logarithmic-transformed lipolysis extent to normalize the response and guarantee positive fitted values with the regression equation. The estimation of the PERT dose that maximises lipolysis extent (i.e., the TOD) and the predictive equations of these models were solved numerically using segmented mixed models with random change points for each food [34].

All the analyses were performed using R software (version 3.3.3), and lme4 (version 1.1-12), nlme (version 3.1-131), and clickR (version 0.3.35) packages. A p-value below 0.05 was considered statistically significant.

3. RESULTS

3.1. Influence of some food extrinsic factors on lipolysis extent

The analysis of the results revealed that the mean values of lipolysis extent at pH 6 and 7 were 60.8 % and 104.74 %, respectively. When considering the bile concentration, experiments performed with 1 mM led to a mean lipolysis extent of 60.37 %, while 99.96 % was reached when bile formulation was 10 mM. Both variables, intestinal pH and bile concentration, had a statistically significant ($p<0.001$) influence on lipolysis. Both increasing bile concentration and increase of intestinal pH had a positive effect on lipolysis (CI 95% [1.28, 1.55] and CI 95% [1.15, 1.39], respectively). In addition, a significant interaction between both factors was found ($p=0.03$, IC95% [-0.28, -0.02]), meaning that food samples digested *in vitro* at intestinal pH 7 and bile concentration of 10 mM had not a lipolysis extent as high as expected with the combination of both effects (CI 95% [-0.28, -0.01]), (**Table 4.19**).

These results evidence that the gastrointestinal conditions of the CF patients do affect the ES activity and thus the lipolysis extent.

Table 4.19. Linear mixed regression model for food extrinsic factors: influence of intestinal pH and bile concentration on lipolysis extent.

Variable		Lipolysis extent (%) Mean (SD)	Exp(Estimate) [CI 95%]	P-value
Intestinal pH	pH 6	60.8 (34.19)	0.35 [0.25, 0.44]	<0.001
	pH 7	104.67 (17.81)		
Bile salts concentration	1 mM	62.37 (35.41)	0.24 [0.14, 0.33]	<0.001
	10 mM	99.96 (21.27)		
Interaction pH7 : 10 mM		-	-0.15 [-0.28, -0.02]	0.033

SD, standard deviation; CI, confidence interval; mM, milimolar

3.2. Influence of enzyme dose on lipolysis extent

In selected foods lipolysis was assessed *in vitro* with different doses of PERT (0, 1000, 2000, 3000 and 4000 LU/g fat) under CF intestinal conditions (pH 6 and Bile 1 mM). According to the results of lipolysis extent as a function of PERT dose, two groups could be differentiated. One food group, consisting of salad with olive oil, pizza, yoghurt and sandwich, reached the maximum lipolysis extent at 2000 LU/g fat, with decreasing lipolysis after further increasing PERT dose (**Figure 4.21a**). On the contrary, the other food group, consisting of milk, bread with butter, cereals and biscuit, exhibited an increasing lipolysis extent when increasing PERT dose above 2000 LU/g fat (**Figure 4.21b**).

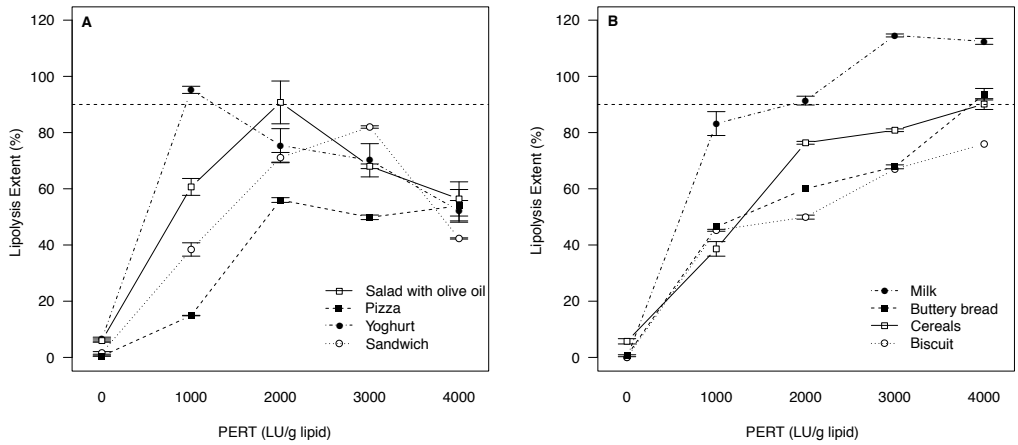


Figure 4.21. Lipolysis extent (%) versus different PERT dose (LU/g lipid) after *in vitro* digestion (intestinal pH 6 and 1 mM bile concentration). The horizontal dotted line represents a 90 % of lipolysis extent target. Figure 4.21 A: foods with an increasing lipolysis extent with dose; Figure 4.21 B: foods with a decreasing lipolysis extent with dose.

3.3. Modelling lipolysis extent under Cystic Fibrosis conditions

A segmented mixed regression model was applied to study and describe the association of lipolysis extent with PERT dose for each food product. By applying this model, it was possible to obtain both, the dose at which the improvement of lipolysis extension changes with dose increase (change points in the lipolysis slopes), and the different slopes after this change point for each food product. It was confirmed that each meal or food product had a change point in the dose of ES from which the slope changes, either because it decreased, i.e. negative slope, (**Figure 4.22a**) or slightly increased, i.e. positive slope (**Figure 4.22b**).

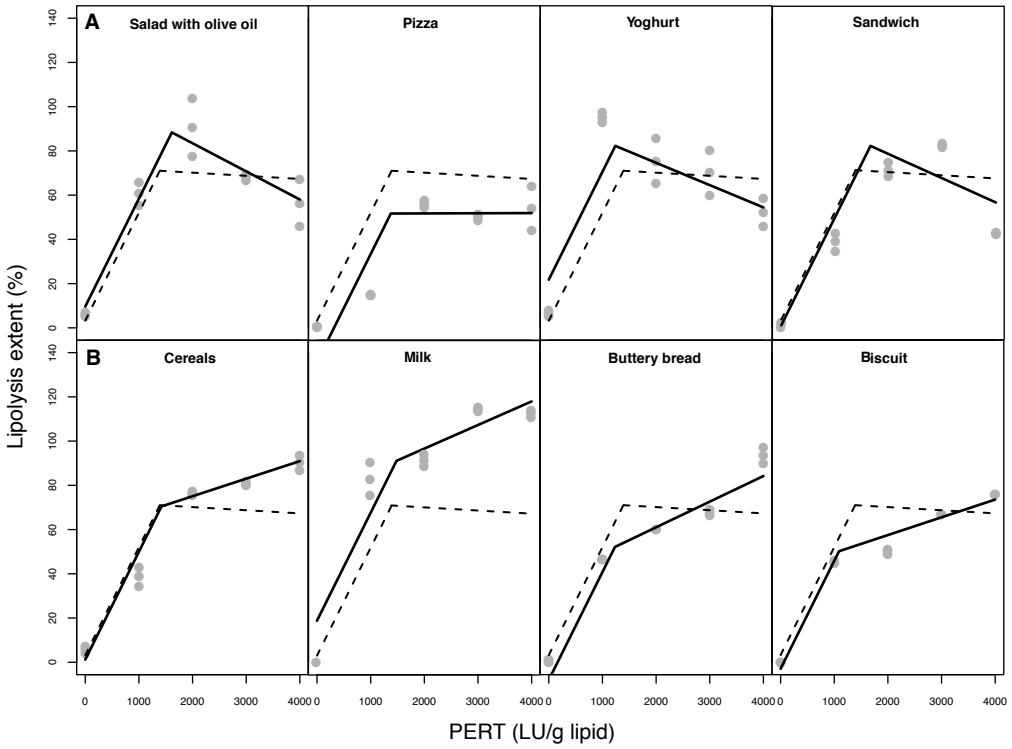


Figure 4.22. Lipolysis extent (%) as a function of PERT dose (LU/g lipid) predicted for each food by applying the segmented mixed regression model. The dotted line represents the evolution considering all the foods together and the solid line shows the estimation for each food individually. Figure 4.22 A: foods with an increasing lipolysis extent with dose; Figure 4.22 B: foods with a decreasing lipolysis extent with dose.

The data modelling provided the equations (**Equation 1**) that enable for the prediction of the optimal dose, allowing for maximum lipolysis, for each food:

Equation 1:
$$Y = \beta_{0i} + \beta_1 * X + \delta_i * (X - \varphi_i) + \varepsilon_i$$
$$i = 1, \dots, 8$$

where,

"Y" is the lipolysis extent (%);

"X" is a continuous variable representing PERT dose (LU/g of lipid);

"i" refers to the type of food (i=1 (salad), i=2 (pizza),…, i=8 (chocolate biscuit)).

For each food i,

"β_0" is the parameter expressing the mean response value at $X = 0$

"β_1" is the slope before the change point. Since there are a very limited number of experimental data in the range between 0 and 1000 LU/g lipid, in which probably different slopes could have been obtained, the same value in all foods assessed is assumed, resulting in a slope of 0.05.

"φ_i" is the dose of PERT at the change point or breakpoint which determines the change in the lipolysis extent trajectory in each food i.

"ε_i" $\sim N(0, \sigma_\varepsilon^2)$, represents the non-explained variance by the regression model.

Thus, to interpret the equation, the "I" function has to be considered, which takes into account the dose (X) and the change point (φ_i), i.e. $(X - \varphi_i)$. It is a logic expression that can equal to "0" (false) when the dose ("X") is lower than the change point, or to "1" when the dose ("X") after the change point is higher. Therefore, if I = 0, the second part of the equation does not apply, since "$\delta_i * (X - \varphi_i)$" would equal to 0.

Thereby, if $\beta_1 + \delta_i > 0$, the slope after breakpoint would be positive and the target 90 % lipolysis extent could be reached. However, if $\beta_1 + \delta_i < 0$ the sign of the slope after changepoint would be negative, then the lipolysis extent could not be increased after the change point, and the maximum lipolysis had been already reached in the change point.

In four out of the eight test foods, a lipolysis extent of 90 could be reached when applying the equations and TOD's could be calculated (**Table 4.20**).

Table 4.20. Summary of lipolysis extent at the change point and theoretical optimal dose (TOD) to obtain lipolysis extent of 90%

Food	Lipolysis extent (%) at change point (φ_i)	Slope sign (δ_i) after change point	TOD (LU) for 90 % lipolysis extent
Salad with olive oil	89.95 % (1613)	negative (-0.013)	-
Pizza	53.73 % (1375)	negative (-0.001)	-
Yoghurt	85.18 % (1240)	negative (-0.011)	-
Ham & cheese Sandwich	81.04 % (1660)	negative (-0.009)	-
Milk	90.49 % (1480)	positive (0.011)	1710
Buttery bread	60.13 % (1230)	positive (0.014)	4000
Cereal	69.22 % (1420)	positive (0.006)	3500
Biscuit	50.03 % (1090)	positive (0.008)	5140

4. DISCUSSION

The approach presented in this study allowed establishing a scientific-based method to analyse the influence of some food intrinsic factors and some physiological conditions on lipolysis, and to predict the theoretical optimal dose of enzyme supplements (TOD) for 8 specific foods in CF specific conditions. The segmented mixed regression models were used to obtain the equations adjusted to the experimental results, which allowed for calculating an optimal enzyme dose to reach a specific lipolysis extent target. The model considered the inherent-to-food properties and the worst gastrointestinal conditions in CF patients (intestinal pH6 and bile 1 mM). In addition, complementary experiments were performed in order to evaluate the influence of other intestinal conditions on lipolysis extent.

The results evidenced that the intestinal pH, along with the bile concentration, were the major determinant factors affecting the process of lipolysis. It is well known that pancreatic lipase activity is higher at pH 7 than at pH 6 [32] and that bile salts contribute to the emulsification process of lipids in the digestive fluids and therefore increasing the interfacial surface of the lipids available for being hydrolysed [35]. This evidence, suggests that proton pump inhibitors can be important as additional therapy to PERT since they would help to increase intestinal pH.

The influence of the inherent-to-food properties was evidenced when assessing the lipolysis extent against PERT dose, at fixed gastrointestinal conditions i.e. pH 6 and bile 1 mM, and "food" considered as a random effect. The segmented regression model showed that some of the assessed foods followed a constantly increasing relationship between PERT dose and lipolysis extent whilst others tended to decrease after a certain dose.

Natural emulsions like milk and yoghurt, with a large interfacial surface (smaller and larger number of available fat molecules) than other solid foods, provide an easier accessibility for the enzymes to the fat globules surface [35,36]. In addition, these matrices are easily disintegrated, favouring the fat molecules' release from the food matrix [37]. Therefore, these two foods were able to reach the highest extents of lipolysis under the simulated conditions. Similarly, the only lipids source in the salad was the added olive oil, this not being trapped into the matrix and thus being very accessible to enzymes (free fat). The same pattern was observed in the buttered bread, where all the lipids came mainly from the butter spread on the bread. Mechanic agitation both in gastric and intestinal stages led to fat phase separation, resulting in a digestion media where lipids are easily emulsified and accessible to the enzymes and not trapped in the food matrix.

On the contrary, we observed that those foods with higher contents of complex carbohydrates were those showing the smallest slopes with increasing PERT dose: sandwich (bread) and pizza (dough). These results may be due to carbohydrates increase the viscosity in the digestion media [13,38] and affect the enzyme-substrate

adsorption process. Several studies assessing nutrient digestion in model systems have reported that the initial structure and composition of the food matrix influences the disintegration mechanism and subsequently the kinetics of nutrients release [39]. For instance, one study assessing proteolysis in yogurt found that the more viscous texture of yogurt could affect food mixing and interactions with enzymes, and contribute to the slightly lower protein digestibility during *in vitro* digestion [33]. More concretely, this phenomenon was also reported in some types of starch [40–42].

The predictive model described two possible patterns in lipolysis extent in relation to PERT doses. Furthermore, reaching the target value, was not dependent on the lipid content of the meals. Thus, milk (3.6 % lipid) and yoghurt (10 % lipid) reached > 90 % lipolysis when using PERT doses of 1000 LU/g lipid, followed by salad (9.4 % lipid) that needed 2000 LU/g lipid. Noteworthy, those foods with the highest percentage of lipids, buttered bread (24 %) and biscuits (27 %), resulted in the highest requirements of PERT per gram of lipid to reach the threshold of 90 % lipolysis.

One limitation of this study is that some inherent-to-food factors (such as nutrient composition or lipid structure in the matrix) have not been taken into account as specific co-variables in the model. The reason for this was the sample size, i.e. eight foods, did not allow for robust analysis when adding variables other than the doses of enzymes. However, these characteristics have been controlled and taken into account in the model by means of the random effect of each food. This effect allows for controlling the non-independence of the data, which means that, for example, each food has its specific change point.

The main strength of this study is, indeed, the inclusion of eight different complex foods as samples for *in vitro* digestion to study lipolysis. Concretely, these foods presented different structural and compositional characteristics, and were tested under different gastrointestinal conditions. Therefore, we consider this integral approach used in the study as appropriate to generate knowledge on lipolysis extent in a multi-variable framework.

Another strength and novelty of this study is the applied statistical model. The mixed segmented model is the most adequate to describe the relationship between PERT and the lipolysis extent, since two linear tendencies, separated by a change point, coexist in the same food product. The model allows for the identification of those foods for whom increasing of PERT dose would lead to a higher lipolysis extent. Besides, the model can be applied to results of other foods undertaking the same experimental design, and it could be assumed that all of them will show one of the two tendencies previously described.

Therefore, the results reported in this paper may have a great potential application in clinical practice in the near future, since they will be applied in an *in vivo* clinical pilot study in paediatric patients with Cystic Fibrosis from six European CF-centres (Part II of this work). Participants will follow a 24-hour menu consisting of the foods we studied, along with the TODs obtained in this part of the study. Stool collections will be used to calculate the coefficient of fat absorption (CFA), which is the clinical correlate of the lipolysis extent. In this way, the prediction model of TODs will be validated *in vivo.*

In conclusion, a first approach towards an evidenced-based method for PERT adjustment has been conducted. This work proves that different intrinsic-to-food factors as well as factors related to the disease-specific gastrointestinal environment will have an effect on the lipolysis extent and consecutively on the TOD. According to our results, the lipid amount in food alone is not an appropriate criterion to adjust the dose of PERT. Besides, each food should be assigned with a specific TOD in order to adjust PERT dosing. These findings warrant further research by the MyCyFAPP working group.

Acknowledgments

Authors of this paper acknowledge the support of the MyCyFAPP Project consortium. We especially thank the contributions of Mieke Boon and Jessie Hulst in reviewing the manuscript.

Financial support

This work was fully funded by the European Union and the Horizon 2020 Research and Innovation Framework Programme (PHC-26-2014 call Self management of health and disease: citizen engagement and mHealth) under grant number 643806.

Conflict of interests

All authors, after having read the journal's policy on conflicts of interest, declare that there are no competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

REFERENCES

1. Farrell, P. M. The prevalence of cystic fibrosis in the European Union. *J. Cyst. Fibros.* **7,** 450–453 (2008).
2. Whitcomb, D. C. *et al.* Pancrelipase delayed-release capsules (CREON) for exocrine pancreatic insufficiency due to chronic pancreatitis or pancreatic surgery: A double-blind randomized trial. *Am. J. Gastroenterol.* **105,** 2276–2286 (2010).
3. Layer, P. & Keller, J. Lipase supplementation therapy: standards, alternatives, and perspectives. *Pancreas* **26,** 1–7 (2003).
4. Sikkens, E. C. M., Cahen, D. L., Kuipers, E. J. & Bruno, M. J. Pancreatic enzyme replacement therapy in chronic pancreatitis. *Best Pract. Res. Clin. Gastroenterol.* **24,** 337–347 (2010).
5. Fieker, A., Philpott, J. & Armand, M. Enzyme replacement therapy for pancreatic insufficiency: Present and future. *Clin. Exp. Gastroenterol.* **4,** 55–73 (2011).
6. Borowitz, D. *et al.* Gastrointestinal outcomes and confounders in cystic fibrosis. *J. Pediatr. Gastroenterol. Nutr.* **41,** 273–285 (2005).
7. Calvo-Lerma, J., Martinez-Barona, S., Masip, E., Fornés, V. & Ribes-koninckx, C. Pancreatic enzyme replacement therapy in cystic fibrosis: dose, variability and

coefficient of fat absorption. *Rev Esp Enf Dig.* **109**, 684-689 (2017).

8. Li, Y., Hu, M. & McClements, D. J. Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: Proposal for a standardised pH-stat method. *Food Chem.* **126,** 498–505 (2011).
9. Schall, JI, Bentley, T, Stallings, V. Meal patterns, Dietary Fat Intake and pancreatic enzyme use in preadolescent children with Cystic Fibrosis. *J. Pediatr. Gastroenterol. Nutr.* **43,** 651–659 (2006).
10. Turck, D. *et al.* ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clin. Nutr.* **35,** 557-77 (2016).
11. Calvo-Lerma, J. *et al.* Nutritional status, nutrient intake and use of enzymatic supplements in paediatric patients with Cystic Fibrosis; a multicentre study with reference to current guidelines. *J. Cyst. Fibros.* **16**, 510-518 (2017).
12. Hunter, E. J. Studies on effects of dietary fatty acids as related to their position on triglycerides. *Lipids* **36,** 655–668 (2001).
13. Fang, X., Rioux, L., Labrie, S. & Turgeon, S. L. Disintegration and nutrients release from cheese with different textural properties during in vitro digestion. *Food Res Int.* **88**, 278-283 (2016).
14. Helbig, A., Silletti, E., Timmerman, E., Hamer, R. J. & Gruppen, H. In vitro study of intestinal lipolysis using pH-stat and gas chromatography. *Food Hydrocoll.* **28,** 10–19 (2012).
15. McClements, D. J., Decker, E. A. & Park, Y. Controlling lipid bioavailability through physicochemical and structural approaches. *Crit. Rev. Food Sci. Nutr.* **49,** 48–67 (2009).
16. Vinarov, Z. *et al.* In vitro study of triglyceride lipolysis and phase distribution of the reaction products and cholesterol: effects of calcium and bicarbonate. *Food Funct.* **3,** 1206–20 (2012).
17. Yen, E. H., Ms, H. Q. & Borowitz, D. Better Nutritional Status in Early Childhood Is Associated with Improved Clinical Outcomes and Survival in Patients with Cystic Fibrosis. *J. Pediatr.* **162,** 530–535.e1 (2013).

18. Rovner, A. J., Schall, J. I., Mondick, J. T., Zhuang, H. & Mascarenhas, M. R. Delayed small bowel transit in children with cystic fibrosis and pancreatic insufficiency: Commentary. *Inflamm. Bowel Dis. Monit.* **14,** 65–66 (2014).

19. Andree, M. *et al.* Malabsorption of bile acids in children with cystic fibrosis. *N. Engl. J. Med.* **289,** 1001–1005 (1996).

20. Bläckberg, L., Hernell, O., Bengtsson, G. & Olivecrona, T. Colipase enhances hydrolysis of dietary triglycerides in the absence of bile salts. *J. Clin. Invest.* **64,** 1303–1308 (1979).

21. Hu, M., Li, Y., Decker, E. A. & McClements, D. J. Role of calcium and calcium-binding agents on the lipase digestibility of emulsified lipids using an in vitro digestion model. *Food Hydrocoll.* **24,** 719–725 (2010).

22. Gelfond, D., Ma, C., Semler, J. & Borowitz, D. Intestinal ph and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. *Dig. Dis. Sci.* **58,** 2275–2281 (2013).

23. Minekus, M. *et al.* A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct. Food Funct* **5,** 1113–1124 (2014).

24. Lesmes, U. & McClements, D. J. Controlling lipid digestibility: Response of lipid droplets coated by β-lactoglobulin-dextran Maillard conjugates to simulated gastrointestinal conditions. *Food Hydrocoll.* **26,** 221–230 (2012).

25. Zhu, X., Ye, A., Verrier, T. & Singh, H. Free fatty acid profiles of emulsified lipids during in vitro digestion with pancreatic lipase. *Food Chem.* **139,** 398–404 (2013).

26. Calvo-lerma, J. *et al.* Innovative approach for self-management and social welfare of children with cystic fi brosis in Europe : development , validation and implementation of an mHealth tool (MyCyFAPP). *Br. Med. J. Open.* **7**, e014931 (2017).

27. Colombo, C., Arlati, S., Curcio, L., Maiavacca, R. & Garatti, M. Effect of Taurine Supplementation on Fat and Bile Acid Absorption in Patients with Cystic Fibrosis. *Scand J Gastroenterol Suppl*. **143**, 151-156 (1988).

28. Norman, A. P. *et al.* Intestinal bile salts in cystic fibrosis. *Arch Dis Child*. **54**, 19-24 (1979).

29. Robinson, P. J., Smith, A. L., Sly, P. D. & Al, R. E. T. Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. *Dig. Dis. Sci.* **35,** 1299–1304 (1990).

30. Youngberg, C. A. *et al.* Comparison of gastrointestinal pH in cystic fibrosis and healthy subjects. *Dig. Dis. Sci.* **32,** 472–480 (1987).

31. Prazeres, D. M. F., Garcia, F. A. P. & Cabral, M. S. Continuous lipolysis in a reversed micellar membrane bioreactor. *Bioprocess Eng.* **10,** 21–27 (1994).

32. González-Bacerio, J., Rodríguez Hernández, J. & del Monte Martínez, A. Lipases: enzymes with potential for the development of immobilized biocatalysts by interfacial adsorption. *Rev. Colomb. Biotecnol.* **12,** 113–140 (2010).

33. Lamothe, S., Azimy, N. & Bazinet, L. Function Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment. *Food Funct.* **5,** 2621–2631 (2014).

34. Muggeo, V. & Muggeo, V. M. R. Segmented mixed models with random changepoints in R Segmented mixed models with random changepoints in R. Working paper (2016). doi:10.13140/RG.2.1.4180.8402

35. Verger, R. Interfacial enzyme kinetics of lipolysis. *Annu. Rev. Biophys. Bioeng.* **5,** 77–117 (1976).

36. Aloulou, A. *et al.* In vitro comparative study of three pancreatic enzyme preparations: Dissolution profiles, active enzyme release and acid stability. *Aliment. Pharmacol. Ther.* **27,** 283–292 (2008).

37. Lamothe, S., Rémillard, N., Tremblay, J. & Britten, M. Influence of dairy matrices on nutrient release in a simulated gastrointestinal environment. *Food Res. Int.* **92,** 138–146 (2017).

38. Brismar, K. Structure of Cooked Spaghetti of Durum and bread wheats. *Starch* **55,** 546–557 (2003).

39. Bornhorst, G. M., Ferrua, M. J. & Singh, R. P. A Proposed Food Breakdown Classification System to Predict Food Behavior during Gastric Digestion. *J Food*

Sci **80,** 924-934 (2015).

40. Resmini, P. & Pagani, M. A. Ultrastructure Studies of Pasta . A Review . *Food Structure* **2,** 1-12 (1983).
41. Ye, X. & Sui, Z. International Journal of Biological Macromolecules Physicochemical properties and starch digestibility of Chinese noodles in relation to optimal cooking time. *Int. J. Biol. Macromol.* **84,** 428–433 (2016).
42. Kim, E. H. *et al.* Effect of Structural and Physicochemical Characteristics of the Protein Matrix in Pasta on In Vitro Starch Digestibility. *Food Biophysics.* **3**, 229–234 (2008). doi:10.1007/s11483-008-9066-7

PAPER 9

EVIDENCE-BASED METHOD TO ADJUST PANCREATIC ENZYME REPLACEMENT THERAPY IN CYSTIC FIBROSIS: PART II, IN-VIVO VALIDATION OF THE IN VITRO MODEL

Joaquim Calvo-Lerma[1,2*], Jessie Hulst[3], Mieke Boon[4], Etna Masip[1], Victoria Fornés-Ferrer[1], Maria Garriga[5], Carla Colombo[6], Ana Andrés[2], Els van der Wiel[3], Ine Claes[4], Mar Ruperto[5], Maria Roca[1], Paula Crespo[1], Anna Bulfamante[6], Sandra Woodcock[3], Kris de Boeck[4] and Carmen Ribes-Koninckx[1] on behalf of MyCyFAPP project

[1] Instituto de Investigación Sanitaria La Fe de Valencia, P.O. Box 46026, Valencia, Spain. Tel (+34) 961246712

[2] Universitat Politècnica de València, Research Institute of Food Engineering for Development, P.O. Box: 46022, Valencia, Spain. Tel (+34) 963879412

[3] Erasmus Medical Center, Sophia Children's Hospital, Rotterdam, the Netherlands.

[4] Pediatric Pulmonology and Cystic Fibrosis Unit, Department of Pediatrics, University Hospitals Leuven, Belgium

[5] Hospital Universitario Ramón y Cajal. Carretera Colmenar Viejo km 9, 100. 28034 Madrid, Spain.

[6] CF Center, Università degli Studi di Milano.Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Via Commenda 9, 20122 Milan (Italy)

* Corresponding author

ABSTRACT

Objectives: To assess the efficacy *in vivo* of a method to adjust the dose of Pancreatic Enzyme Replacement Therapy (PERT) in Cystic Fibrosis (CF) extrapolated from previous *in vitro* digestion studies (theoretical optimal dose, TOD). Secondly, to assess how individual patient characteristics influence the expected coefficient of fat absorption (CFA) and thus to identify an individual correction factor to improve TOD.

Methods: A prospective interventional study in 43 paediatric patients with CF from 5 European CF centres. They followed a 24h fixed diet with TOD for each meal. Colorimetric dyes were used at start and end of the fixed diet and a continuous faeces

collection was performed, so identification of the faeces corresponding to the fixed diet was possible. Beta regression models were applied to assess the associations of individual patient characteristics with the CFA.

Results: Median CFA was 90% (84, 94% 1st, 3rd Q.) with no significant differences among centres. Transit time was positively associated with CFA (p = 0.007), but no statistical associations were found with and age, gender, mutation or BMI. Regression model showed no improvement of the *in vitro* predicted TOD when taking individual patient characteristics into account.

Conclusion: Strict adherence to the TOD PERT for a prescribed meal, led to median CFA levels at the clinical target of 90% with a low variability between patients. The proposed method can be considered as a first approach for an evidence-based method in PERT dosing based on food characteristics. Results have to be confirmed in free dietary settings.

Keywords: cystic fibrosis; pancreatic insufficiency; pancreatic enzyme replacement therapy lipase; digestion; coefficient of fat absorption

1. INTRODUCTION

Pancreatic insufficiency is associated to Cystic Fibrosis (CF) in around 85-90 % of the patients (REF). Pancreatic Enzyme Replacement Therapy (PERT) is the indicated treatment to compensate for maldigestion and malabsorption of nutrients [1], and aims at maintaining or achieving an adequate nutritional status. Over the last decades, several studies have revealed an imperative need of evidence-based guidelines to adjust the dose of PERT for every meal [2–4], without any successful attempt up to date, to our knowledge.

Determination of fat in faeces and calculation of the coefficient of fat absorption (CFA) have been used as the golden standard method to assess PERT dosage efficacy [5]. The CFA reflects the amount of fat excreted in faeces compared to the dietary intake of fat. Lately, an enormous variability in the response to PERT among patients

has been confirmed, with no clear association between CFA and PERT dose [5,6]. Moreover, a wide intra- and inter-patient range of PERT dose (LU/g fat) in a multicentre observational study was showed [7].

Recent research has identified the important influence of foods' characteristics on the process of lipolysis [4,8–10]. Few investigations have considered integration of the specific food characteristics of a wide range of foods to estimate the dose of PERT and no trials have been performed to study this *in vivo*.

The main goal of the Horizon 2020 MyCyFAPP Project is to establish an evidence-based method to identify the optimal dose of PERT in CF, which will allow for patients' self-management of this therapy by means of a mobile app [11]. This project included an *in vitro* digestion study of foods under standard CF simulated gastrointestinal conditions (see PAPER on Part I) in order to determine the theoretical optimal PERT dose (TOD) per food product, based on food characteristics. The modelling of results confirmed that not only the amount of fat, but also the food characteristics – e.g. amount of other nutrients or matrix properties - lead to very variable PERT doses requirement for optimal digestion of food products with the same total amount of fat.

Next, the project aims to evaluate the efficacy of the TODs, when applied *in vivo* to patients. We hypothesized that an individual correction factor (ICF) would be identified for each patient to correct for individual patient characteristics (gender, age, mutation type, transit time). Correction of the TOD by ICF could result in the individual optimal dose (IOD), leading to the optimal dose of PERT needed to obtain an optimal CFA *in vivo*.

Therefore, the first aim of the present study was to assess the *in vivo* efficacy of an evidence-based prediction model to adjust PERT obtained by means of an *in vitro* digestion experimental setting. Secondly, we aimed at identifying an individual correction factor (ICF), based on individual patient characteristics, representing the difference between the *in vitro* and *in vivo* scenarios. Therefore, in a prospective interventional pilot study, we evaluated the variability in CFA between patients after

consumption of a predefined 24 hours diet in combination with the TOD of PERT, to identify this ICF.

2. METHODS

2.1. Subjects

Paediatric patients with CF followed up as outpatients in 5 European CF centres participating in the MyCyFAPP project: Madrid (Spain), Valencia (Spain), Milan (Italy), Leuven (Belgium) and Rotterdam (The Netherlands) were considered for enrolment.

Inclusion criteria required a confirmed diagnosis of CF (a documented sweat chloride ≥ 60 mEq/L and a documented genotype with two disease-causing mutations in the CFTR gene), age between 2 and 18 years, having pancreatic insufficiency (faecal elastase < 200 mcg/g faeces), and need for treatment with PERT, having a stable clinical status at least two weeks before signing the informed consent and patients' having the capacity and willingness to fulfil the test meals and the faeces collection.

The exclusion criteria were the presence of an acute infection associated with decreased appetite or fever at the time of the run-in visit, acute abdominal pain, severe cholestasis (direct bilirubin increase >2 mg/dL with respect to normal limit for age), FEV_1 <40% predicted, severe hypoalbuminemia (<2.5g/ml in blood), hospitalisation admissions or intravenous antibiotics <2 weeks before signing the informed consent, changes in the usual treatment (prokinetics, proton pump inhibitors, H_2 blockers and antibiotics) <2 weeks before signing the informed consent, presence of alterations that could endanger the safety of the patient and hypersensitivity or adverse reactions to the enzymatic supplements. Lung transplantation was considered an exculusion criterion too.

2.2. Study design

We carried out a multicentre, prospective, interventional study. All participants followed a fixed, predefined study diet composed by the previously *in vitro* digested

foods, in combination with a fixed predefined dose of PERT based on the TOD obtained in the *in vitro* digestion studies (**Figure 4.23**).

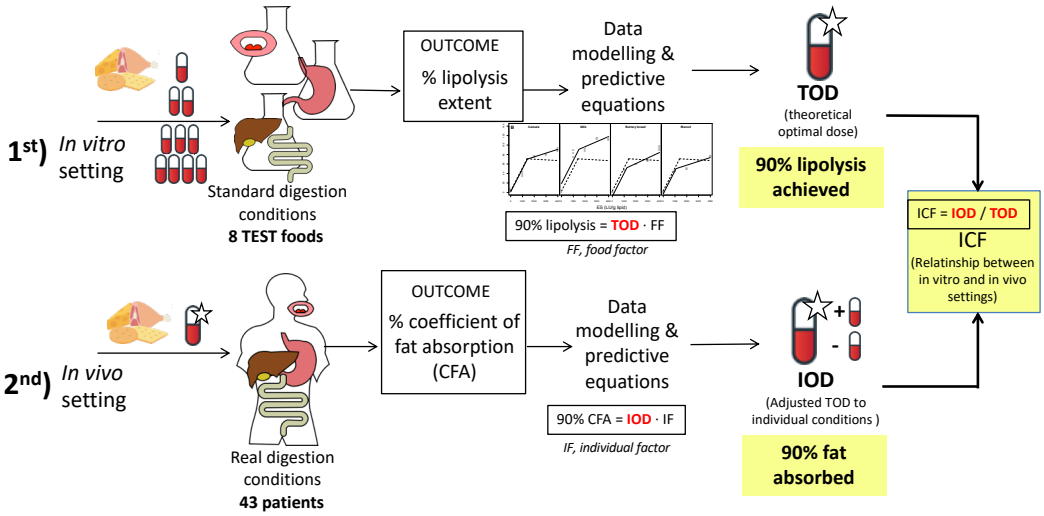


Figure 4.23. Overview of the combined *in vitro* and *in vivo* approach to validate a new evidence-based predictive model to optimally adjust PERT dose, adapted to foods' and patients' characteristics. First a selection of 8 meals were digested *in vitro* under standard CF conditions, and the % of lipolysis was obtained for different doses. Modelling of the % lipolysis extent allowed to predict the theoretical optimal dose for each meal (TOD), which took into account the food characteristics. Second, in the *in vivo* study the same 8 meals were digested *in vivo* under real conditions along with their TODs, and the % of fat absorption was measured in all the participating patients. Modelling of the CFA allowed to obtain how different TOD should have been in order to achieve a 90% of CFA (the equivalent outcome to the *in vitro* setting). The Individual correction factor (ICF) allowed for quantifying the relation between the *in vitro* and the *in vivo* settings. *TOD, theoretical optimal dose; IOD, individual optimal dose; ICF, individual correction factor.*

Patients were provided with a study protocol, which included a detailed instruction handbook to perform the study and as a diary (**Annex 6**). Structured as a 3-day diary, the study protocol showed pictograms with the exact amount of each food item to be registered by the patient or the caregiver and the corresponding PERT dose for each meal. Also questions about compliance after it were asked. A pillbox was provided with the predefined TOD dose of PERT and the colorimetric markers. A faeces

registry form was included at the end of each day, where patients could indicate the number of faeces, its Bristol stool scale classification [12], the colour and the time. Patients were asked to collect faeces corresponding to the digestion of the study menu: this was performed by using colorimetric markers at the start and after the end of the study diet to enable identification of the faeces corresponding to it.

At the study visit, patients' individual characteristics were collected as study variables: age, gender, intake of proton pump inhibitor, BMI z-score, and severity of mutation, this last being defined as Class I, Class II and Class III [13]. The study protocol was approved by the ethical committee of each CF centre. All parents and patients were informed about the purpose and ultimate aim of the study and gave written informed consent.

2.2.1. The test diet and study doses

The test diet followed by the patients consisted of 9 meals distributed in a 24h diet (6 meals) plus a washout period before and after it (3 meals) to minimise the impact of the regular and uncontrolled normal diets of the participants in the faeces analysis. All the meals included in the test diet were previously digested *in vitro* and the corresponding TODs were estimated (Part I). **Figure 4.24** provides the overview of the study timeline per patient.

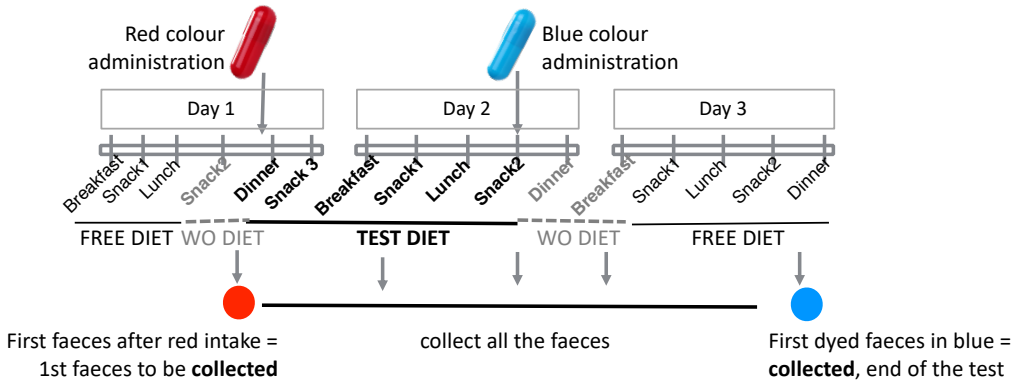


Figure 4.24. Overview of the study. The study started at day 1 afternoon with a washout diet including a fixed PERT dose (snack 2) and the administration of the red marker. From then on, and during day 2 patients followed the test diet with the fixed PERT dose, until the afternoon snack on day 2, that was taken

in combination with the blue markers. The dinner of day 2 and the breakfast of day 3 conformed the washout diet after the test menu, also with a fixed PERT dose. Faeces had to be collected from the moment of the red colour intake until the first blue-dyed deposition was found. For the faeces analysis, researchers selected the samples comprised from the 1st red deposition to the 1st blue. *WO, washout*.

In the design of the meals the following considerations were taken into account for the feasibility and to avoid country-related potential bias: meals had to fit all the nutritional habits according to previous results of our group, international brands had to be used in order to achieve the same nutritional composition and easy cooking techniques were to be used. Four energy levels were proposed so that patients of all ages could participate: 1622, 1832, 2194 and 2573 kcal/24h. In all of them, the macronutrient distribution was approximately 40% lipids, 40% carbohydrates and 20% protein, according to the nutrition guidelines [1]. In each level, the foods were the same, but the amounts varied (**Annex 7**) and TOD dose was adapted proportionally. Families and patients were allowed to choose the level that most appropriately fitted the appetite and habits of the participant.

2.2.2. Faeces collection and analysis

Detailed instructions were given to minimise the error in the CFA calculations. From the moment of the red marker intake, all the faeces had to be collected individually into separate plastic bags. Fecotainers® (AT Medical BV, The Netherlands) were provided to the patients to ease the collection. This process was repeated until the blue colour was identified in the faeces.

The faeces were shipped from all the participating centres to the central lab in Instituto de Investigación Sanitaria La Fe (Valencia, Spain) for analysis. Homogenisation of all the samples of each child was carefully carried out, only including the faeces corresponding to the test menu: the samples without red colour that were collected before the appearance of red-dyed faeces were discarded. Samples considered for the analysis comprised those collected between the 1st red and the 1st blue-dyed, both

included. Then household stirrers 750 W (Braun MQ735) were used to mix the faeces contained in the original recipients until complete homogenisation was observed (5 minutes per sample approx., depending on the consistency and volume). Careful cleaning of the equipment was performed between samples. Then the homogenised sample was aliquoted in 10g recipients and kept under freezing conditions (-20 ºC). None of the protocols for homogenisation available in the literature were applied because the method was vaguely described or not available at all [14–17] and also because there is no standardised method.

Quantitative faecal fat analyses were carried out by the infrared spectroscopy technique, the golden standard to perform fat in faeces analyses at the Reference Laboratory (Barcelona, Spain). Random selected samples (n=10) were used to assess the homogenisation protocol developed. Test results were highly reproducible (CV <15%) between two different aliquots of the same homogenised sample

The CFA was calculated as the percentage of fat excreted in faeces (considering the faeces belonging to the test diet) compared to the fat in the test diet. This parameter is considered equivalent to the % lipolysis extent used in the *in vitro* setting. The transit time was calculated as the time between the ingestion of the red capsule and the moment the first red faeces appeared.

The Bristol Stool Scale was used for patients to report the faeces consistency. This scale is used to assess gastrointestinal complications. Ranging from type 1 to type 7, where 1 is extreme constipation and 7 extreme diarrhoea, the optimal types are 3-4.

2.3. Calculations and statistical analyses

Sample size calculation was performed assuming that differences in undigested fat between in-vitro and in-vivo digestions would range from 0g to 18g, with a mean value of 9g and a standard deviation of 3g (according to a retrospective review of clinical data from patients from the participating centres [6]). With these values, 41 individuals would be needed to achieve a precision of ±1g of fat (95% CI length of 2) in

the determination of the differences in undigested fat between in-vitro and in-vivo values. Assuming a drop-out of 20% of the recruited patients, the study sample size should be 50 subjects.

For the descriptive analysis, data were summarised using mean (standard deviation) or median (1st, 3rd Q.) in the case of continuous variables and with relative and absolute frequencies in the case of categorical variables.

A beta regression model was applied to study the association between the CFA and the selected study variables: age, PERT dose (i.e. TOD), use of proton pump inhibitor (PPI) and transit time.

The results of the beta regression model can be interpreted with the exp(estimate) and the CI 95%. An exp(estimate) >1 means that the variable is positively associated with the response variable and the higher the value, the higher the effect. Complementarily, the confidence intervals that do not contain 1 are those significantly associated with the response variable.

This model was applied to calculate the optimal dose of enzymes for each patient (IOD) to reach a target of 90% lipolysis extent (i.e., CFA), according to the individual patient characteristics by means of the equation of the model (**Equation 1**).

Equation 1:

$$g(\%CFA) = \beta_0 + (\beta_1 \cdot transit\ time) + (\beta_2 \cdot IOD) + (\beta_3 \cdot age) + (\beta_4 \cdot PPI\ intake)$$

where g (·) is the logit function of the beta regression, IOD is the individual optimal dose of enzymes and PPI is proton pump inhibitors. β_i , $i = 0, \dots, 4$ are the model parameters.

Therefore, to calculate the IOD for each patient to reach the clinical target of 90% CFA, this equation should be solved:

$$IOD = \frac{g\left(90\%_{clinical\ target\ CFA}\right) - \beta_0 - (\beta_1 \cdot transit\ time) - (\beta_3 \cdot age) - (\beta_4 \cdot PPI\ intake)}{\beta_2}$$

Then, the relationship between the IOD and the actual TOD taken by the patient in the study conformed the individual correction factor (ICF). The ICF is thus the ratio that reflects the relationship between the *in vitro* and the *in vivo* settings of the digestion (**Equation 2**). This ratio, thus, expresses how different the TOD has to be, either higher or lower, than the one predicted *in vitro*, encompassing the individual characteristics that differ from those standard characteristics simulated *in vitro*.

Equation 2:

ICF = (IOD) / (TOD)

Where ICF is the individual correction factor; IOD is the in vivo optimal dose obtained by Equation 1 in which patients characteristics were considered, in order to reach a 90% of CFA; and TOD is the theoretical optimal dose of enzymes obtained in vitro, which was actually taken by the participants during the study.

A mixed ordinal regression model was carried out to study the association between CFA and the response Bristol Stool Scale. The model was extended with the "Patient" variable as a random effect with a random intercept to correct for the non-independence of the data since there was more than one faeces sample per patient.

Patients' compliance with the study protocol was defined as intake of fat and PERT as compared to the indicated amount (%).

All the analyses were performed using R software (version 3.3.3), and lme4 (version 1.1-12), nlme (version 3.1-131), ordinal (version 2016.6-28), betareg (version 3.1-0), clickR (version 0.3.35) packages. A p-value below 0.05 was considered statistically significant.

3. RESULTS

A total of 54 patients were enrolled in the study between April and June 2017: 9 from Madrid, 12 from Valencia, 5 from Milan, 13 from Leuven and 9 from Rotterdam.

Eleven participants dropped out due to stress for defecating in bags (n= 5), because of severe infections that were not related to the study (n= 2) and antibiotic use (n= 2), so the final number of participants was 43.

3.1. Patients characteristics and descriptive results

Table 4.21 shows the patient characteristics of the study population. Median age, BMI z-score and transit time were 9.4 years, -0.02 SD and 28.5h respectively. The median PERT dose was around 2599 LU/g fat, according to the indications of PERT dose in the protocol for the participants. Compliance with the test diet and the test PERT dose (TOD) were 98.4 and 100% respectively. None of the collected variables presented statistical differences among centres. None of the recruited patients reported adverse events related to the study nor symptoms or complaints with the exception of one patient who experienced an episode of acute diarrhoea.

3.1.2. The coefficient of fat absorption CFA

Median CFA was 90.0% considering all the centres together. When analysing results of each centre individually the same distribution was found in all (**Figure 4.25**), with values ranging from 83.7% (1st quartile) to 94.4% (3rd quartile) and no significant differences in CFA between centres (p = 0.60).

There were three outliers: one in Madrid (CFA 53%) due to the wrong collection of samples; and two in Leuven, one was ill suffering from diarrhoea with reported Bristol stool scale numbers of 7 and transit time of 12h (CFA 61%) and for the other the low compliance with the protocol was associated to the low 68% CFA.

3.1.3. Bristol Stool Scale and CFA

Most of the faeces were identified by the participants as Bristol stool scale types 3 and 4 (normal) and 2 (constipation) as shown in **Figure 4.26**. No association was found between CFA and Bristol Stool Scale number (p >0.05).

Table 4.21. Patients' characteristics and results of the pilot study

	Madrid (n = 9)	Valencia (n = 10)	Milan (n = 5)	Leuven (n = 12)	Rotterdam (n = 7)
Age (years) *Median (1st, 3rd Q.)*	12.1 (9.3,14.5)	7.5 (6.9,16.3)	8.2 (6.2,11.6)	7.4 (7.2, 8.5)	10.1 (9.6, 12.9)
Male, *n (%)*	6 (67)	5 (50)	2 (40)	7 (58.3)	3 (43)
BMI z-score *Median (1st, 3rd Q.)*	0.01 (-0.08, 0.07)	-0.24 (-1, 0.41)	-0.26 (-0.94, 0.58)	0.08 (-0.5, 0.3)	-0.02 (-0.2, 0.66)
CFA (%) *Median (1st, 3rd Q.)*	91.2 (84.7, 93.9)	88.8 (84.9, 92.8)	94.1 (84.7, 95.2)	88.1 (79.7,91.47)	89.9 (87.5, 94.3)
Total fat in faeces (g) *Median (1st, 3rd Q.)*	7.8 (6.7, 9.5)	8.1 (4.85, 10.62)	5.9 (3.5, 9.6)	10.35 (7.2, 15.8)	9.45 (5.1, 11.1)
Transit time (h) *Mean (SD)*	24.2 (15.1)	25.9 (12.9)	30.4 (19.2)	30.0 (10.7)	32.1 (13.2)
TOD (LU/g fat) *Median (1st, 3rd Q.)*	2271 (2243, 2540)	2802 (2315, 2866)	2509 (2000, 2579)	2706 (2557, 2786)	2624 (2602, 2977)
LU/ kg weight / day *Median (1st, 3rd Q)*	6008 (4632, 7173)	5531 (4680, 5927)	5608 (4688, 7073)	7917 (5642, 8904)	6752 (5480, 7494)
Compliance with test diet (%) *Median (1st, 3rd Q.)*	108.7 (97.0, 112.2)	94.7 (87.0, 105.5)	99.9 (93.2, 100.0)	100.1 (92.5, 106.4)	97.0 (88.8, 105.3)
Compliance TOD (%) *Median (1st, 3rd Q.)*	100 (89.4, 100)	94.11 (84.7, 100)	85.5 (62.7, 100)	100 (100, 103.4)	100 (94.4, 100)

Compliance exceeding 100% means that amount of fat or enzymes was higher than the indicated in the protocol

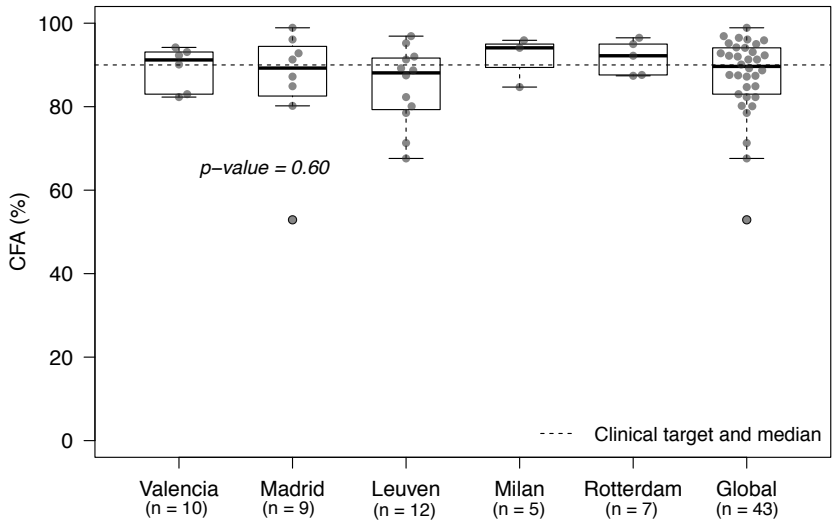


Figure 4.25. Results of the coefficient of fat absorption (CFA) obtained in the participating centres. The horizontal dotted bar represents the median CFA of the total population, coinciding with the clinical target of 90% . Boxplots represent the median and the 1st and 3rd quartiles in all the centres.

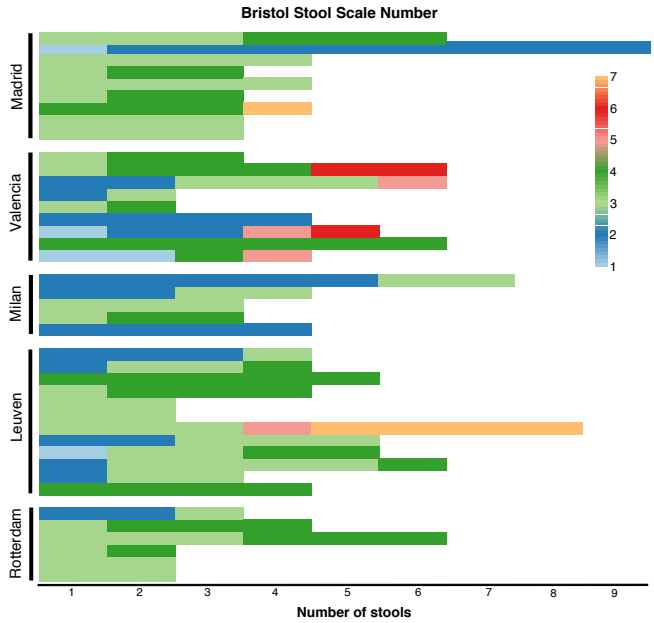


Figure 4.26. Representation of number of faeces per patient and centre and the Bristol stool scale type assigned to each individual deposition by the patient or caregiver (one patient per row). Faeces samples correspond to the study period, from the red to the blue coloured deposition.

3.3. A beta regression model to predict the individual correction factor

Table 4.22 shows the parameters of the beta regression model including the definitively selected variables. The variables type of mutation and BMI z-score considered firstly, were discarded in the model as the mutation was severe in all patients and the BMI z-score did not show any effect in alternative models explored previously. As for the selected variables, the CFA was significantly associated with the transit time (p 0.007): the longer the transit time, the higher the CFA (CI 95% [1.177, 2.797]). The CFA was not significantly associated with PPI intake, TOD and age (p 0.09, p 0.14 and p 0.62 respectively).

Table 4.22. Beta regression model to assess the influence of the study variables on CFA, including the dose of enzymes (TOD) and the individual factors intake of proton pump inhibitors (PPI), age and transit time

	(exp)Estimate	**Confidence Interval CI 95%**	**p-value**
(Intercept) (β_0)	2.839	[0.223, 36.147]	0.42
TOD (β_2)	0.999	[0.998, 1.000]	0.13
PPI intake (β_4)	1.367	[0.885, 2.115]	0.09
Age (β_3)	1.013	[0.961, 1.069]	0.62
Transit time (β_1)	1.815	[1.177, 2.797]	**0.007**

The resulting equation (**Equation 3**) of the beta regression model was applied to predict the IOD, which is what the PERT dose should have been in order to achieve a 90% CFA instead of the CFA value actually obtained with the TOD. This change would have been determined by the individual patient characteristics (transit time, age and PPI intake).

Equation 3:

$$g(\%CFA) = 1.043 + (0.596 \cdot transit\ time) - (0.0004 \cdot IOD) + (0.013 \cdot age) + (0.312 \cdot PPI\ intake)$$

where g (·) is the logit function of the beta regression, IOD is the individual optimal dose of enzymes and PPI is proton pump inhibitors

Then **equation 3** was applied in order to determine the relationship between the TOD and the IOD, or the relationship between the *in vitro* and the in vivo setting. This relationship would have conformed the individual correction factor (ICF). The ICF median was found to be 0.95 (0.92, 1.01) in all the cases, meaning that optimal dose obtained *in vitro* (TOD) was equivalent to the required dose in vivo (IOD).

4. DISCUSSION

Through the present study we have moved from an *in vitro* experimental set-up to an *in vivo* setting to evaluate the model for adjusting the dose of Pancreatic Enzyme Replacement Therapy (PERT) in Cystic Fibrosis (CF). *In vitro* digestions allowed for the estimation of a TOD of enzymes for different meals and foods, and in this pilot study the validity of such an approach was assessed. We had hypothesised that when applying the TOD in vivo, specific individual corrections of it would be needed because of individual subject's characteristics. However, we found that these characteristics did not imply a modification of the dose predicted in the lab, being the relationship between both settings close to 1. In other words, our results suggest that the food characteristics are the determinants of the requirements of PERT, and not the individual patient characteristics we have assessed.

In our clinical setting, using the TOD of PERT, the median CFA was found to be 90%, which is slightly higher than in previous studies, and comparable to others without manipulation of PERT dose [6,18]. In a similar CF-population from Valencia a mean CFA of 86% was reported [5]. In this study a high variability between patients, and within patients (differences in CFA in 3 consecutive days) along 3 visits in a year [6] was found and attributed to the uncontrolled diet and dose of enzymes for the specific meals. In contrast, in the present study with a controlled diet, we found a lower between-patients variability (84 to 94% 1st, 3rd Q.), which suggests that the TOD was robust. Another indicator of the adequacy of the application of the TOD is that most of the faeces were scored types 3-4 on the Bristol stool scale, which is the value indicating the absence of gastrointestinal complications. In addition, no symptoms or complications

were reported by the patients so overall there were no severe gastrointestinal alterations during the study period.

As for the multivariate model, the equation described by the included parameters conforming it was supposed to be used to calculate the individual optimal dose. With the equation of the model, according to the parameters and the individual characteristics of the patients, such as age and PPIs intake, the optimal LU/g fat (IOD) could be calculated in order to obtain the desired CFA of 90%. However, these characteristics turned out to have a very low overall effect, so the TOD and the IOD were almost the same in all the cases (i.e. the ICF was around 1 for all the patients). Transit time was the only variable showing a significant effect. The higher CFA when having a longer gastrointestinal transit time could be attributed to more time for lipid digestion and absorption. However, the transit time is difficult to influence or control in clinical practice. To our knowledge, there are no other studies in the literature assessing this association. Then, the positive (but not significant) effect of proton pump inhibitors has been repeatedly reported in patients with CF [19,20]. The rationale behind it is that PPI's inhibits the acid secretion in the stomach and thus higher pH values are achieved for the intestinal digestion phase. Lipase activity is directly associated with the intestinal pH [21]. This phenomenon was clearly observed in the *in vitro* digestion experiments (Paper part 1).

There are several limitations in this study. First, the number of variables included in the statistical model were limited due to the low patient number. Besides, the model relies on non-significant associations, thus its validity as a predictive model to calculate the ICF in every patient is limited. Nonetheless, according to the biostatistics results, if more patients had been included, the same results distribution was to be expected as long as the protocol was strictly followed. Hence, the multivariate model would not have allowed for an IOD prediction either. Other possibly interesting variables representing patients' individual characteristics that could have provided clearer explanation of the results could be intestinal pH and bile salts concentration, but because of the complexity and invasiveness of the methods to

collect this information, we did not include these variables in the framework of the study. Despite this reasoning, most of the patients had a high CFA value, this being the main reason for no statistical association of CFA and other study variables (apart from transit time). Another limitation of the study was the complexity of the protocol for the participants, although the compliance was nearly 100%.

In contrast, the strengths of the present study rely on the robust methodology. To our knowledge, there are no studies assessing PERT dose and CFA with such an accurate and detailed protocol for faeces collection, homogenisation of faeces samples, analysis and CFA calculation. Besides, to our knowledge this is the first study assessing CFA conducted with a fixed diet and a fixed dose of enzymes, this last being in addition estimated from an evidence-based approach.

Overall, when strictly following the protocol (including test menu, TODs and faeces collection) and in the absence of adverse events that interfere with fat digestion and/or absorption, the use of TODs are precise enough to obtain good CFA values. We have no arguments to assume that other factors related to the subjects with a relevant influence on the ICF could be found.

Our findings may have a great impact on clinical practice. The newly developed evidence-based method to adjust PERT could be implemented in the clinical practice to support clinicians and patients' decision on PERT dose. We showed that the digestion of fat is more dependent on the food characteristics than on the patient characteristics. Ideally, a dose tailored at each single meal could be foreseen. For this implementation, however, a supporting system would be required. In the next step of the MyCyFAPP project, a multicentre clinical trial will assess the influence of using a mobile app, including a calculation algorithm of the PERT dose based on a database of TODs, on health-related outcomes.

In conclusion, we have made a step ahead in the understanding of PERT dosing and we have set up an evidence-based method to adjust PERT to food characteristics in CF. Although we expected that optimal dosing of PERT would be based on the theoretical optimal obtained *in vitro* and further improved by adding an individual

correction factor thereafter, this could not be defined. However, the *in vitro* predicted TODs led to good CFA results *in vivo*, and can therefore be reliably used as a first approach.

Acknowledgments

Authors of this paper acknowledge the support of the MyCyFAPP Project consortium. We especially thank the participation and the effort of the patients involved in the study and their families.

Financial support

This work was fully funded by the European Union and the Horizon 2020 Research and Innovation Framework Programme (PHC-26-2014 call Self management of health and disease: citizen engagement and mHealth) under grant number 643806.

Conflict of interests

All authors, after having read the journal's policy on conflicts of interest, declare that there are no competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

REFERENCES

1. Turck, D. *et al.* ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clin. Nutr.* **35,** 557-577 (2016).
2. Borowitz, D. *et al.* Gastrointestinal outcomes and confounders in cystic fibrosis. *J. Pediatr. Gastroenterol. Nutr.* **41,** 273–285 (2005).
3. Schall, JI, Bentley, T, Stallings, V. Meal patterns, Dietary Fat Intake and pancreatic enzyme use in preadolescent children with Cystic Fibrosis. *J. Pediatr. Gastroenterol. Nutr.* **43,** 651–659 (2006).
4. Li, Y., Hu, M. & McClements, D. J. Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: Proposal for a standardised

pH-stat method. *Food Chem.* **126,** 498–505 (2011).

5. Woestenenk, J. W., Ent, C. K. Van Der & Houwen, R. H. J. Pancreatic Enzyme Replacement Therapy and Coefficient Cystic Fibrosis. **61,** 355–360 (2015).
6. Calvo-Lerma, J., Martinez-Barona, S., Masip, E., Fornés, V. & Ribes-koninckx, C. Pancreatic enzyme replacement therapy in cystic fibrosis: dose, variability and coefficient of fat absorption. *Spanish J. Gastroenterol.* **109,** 684-689 (2017).
7. Calvo-Lerma, J. *et al.* Nutritional status, nutrient intake and use of enzymatic supplements in paediatric patients with Cystic Fibrosis; a multicentre study with reference to current guidelines. *J. Cyst. Fibros.* **16,** 510-518 (2017).
8. Hunter, E. J. Studies on effects of dietary fatty acids as related to their position on triglycerides. *Lipids* **36,** 655–668 (2001).
9. Helbig, A., Silletti, E., Timmerman, E., Hamer, R. J. & Gruppen, H. In vitro study of intestinal lipolysis using pH-stat and gas chromatography. *Food Hydrocoll.* **28,** 10–19 (2012).
10. Fang, X., Rioux, L., Labrie, S. & Turgeon, S. L. Disintegration and nutrients release from cheese with different textural properties during in vitro digestion. *Food Res Int.* **88,** 276-283 (2016)
11. Calvo-lerma, J. *et al.* Innovative approach for self-management and social welfare of children with cystic fi brosis in Europe : development , validation and implementation of an mHealth tool (MyCyFAPP). *Br. Med. J. Open.* **7,** e014931 (2017).
12. Riegler, G. & Expósito, I. Bristol scale stool form. A still valid help in medical practice and clinical research. *Tech. Coloproctol.* **5,** 163–164 (2001).
13. Boeck, K. De & Amaral, M. D. Progress in therapies for cystic fi brosis. *Lancet Respir.* **2600,** 1–13 (2016).
14. Koumantakls, G. & Radciltf, F. J. EstimatingFat in Feces by Near-Infrared ReflectanceSpectroscopy. **33,** 502–506 (1987).
15. Rivero-marcotegui, A., Olivera-olmedo, E., Palacios-sarrasqueta, M., Grijalba-uche, A. & Garcı, S. Water , fat , nitrogen , and sugar content in feces : reference

intervals in children. **1544,** 1540–1544 (1998).

16. Korpi-steiner, N. L., Ward, J. N., Kumar, V. & Mcconnell, J. P. Clinica Chimica Acta Comparative analysis of fecal fat quantitation via nuclear magnetic resonance spectroscopy (1 H NMR) and gravimetry. *Clin. Chim. Acta* **400,** 33–36 (2009).
17. Dorsey, J. *et al.* Fat malabsorption in cystic fibrosis: comparison of quantitative fat assay and a novel assay using fecal lauric/behenic acid. *J. Pediatr. Gastroenterol. Nutr.* **50,** 441-446 (2010).
18. Woestenenk, J. W., Ent, C. K. Van Der & Houwen, R. H. J. Pancreatic Enzyme Replacement Therapy and Coefficient Cystic Fibrosis. *J. Pediatr. Gastroenterol. Nutr.* **61,** 355–360 (2015).
19. Proesmans, M. & De Boeck, K. Omeprazole, a proton pump inhibitor, improves residual steatorrhoea in cystic fibrosis patients treated with high dose pancreatic enzymes. *Eur. J. Pediatr.* **162,** 760–763 (2003).
20. Vecht, J., Symersky, T., Lamers, C. B. & Masclee, A. A. Efficacy of lower than standard doses of pancreatic enzyme supplementation therapy during acid inhibition in patients with pancreatic exocrine insufficiency. *J. Clin. Gastroenterol.* **40,** 721–725 (2006).
21. Robinson, P. J., Smith, A. L., Sly, P. D. & Al, R. E. T. Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. *Dig. Dis. Sci.* **35,** 1299–1304 (1990).

PAPER 10

ASSOCIATION BETWEEN FAECAL PH AND COEFFICIENT OF FAT ABSORPTION IN CHILDREN WITH CYSTIC FIBROSIS ON A CONTROLLED DIET AND DOSE OF PANCREATIC ENZYME REPLACEMENT THERAPY

Joaquim Calvo-Lerma[1,2*], Maria Roca-Llorens[1], Natalia Marco[1], Mieke Boon[3], Jessie Hulst[4], Maria Garriga[5], Carla Colombo[6], Victoria Fornés-Ferrer[1], Ine Claes[3], Etna Masip[1], Ana Andrés[2], Kris de Boeck[3] and Carmen Ribes-Koninckx[1] on behalf of MyCyFAPP project

* Corresponding author
Joaquim Calvo Lerma
Instituto de Investigación Sanitaria La Fe de Valencia, P.O. Box 46026, Valencia, Spain. Tel (+34) 961246712
joaquin_calvo@iislafe.es

1 Instituto de Investigación Sanitaria La Fe de Valencia, P.O. Box 46026, Valencia, Spain. Tel (+34) 961246712
2 Universitat Politècnica de València, Research Institute of Food Engineering for Development, P.O. Box: 46022, Valencia, Spain. Tel (+34) 963879412
3 Pediatric Pulmonology and Cystic Fibrosis Unit, Department of Pediatrics, University Hospitals Leuven, Belgium
4 Erasmus Medical Center, Sophia Children's Hospital, Rotterdam, the Netherlands.
5 Hospital Universitario Ramón y Cajal. Carretera Colmenar Viejo km 9, 100. 28034 Madrid, Spain.
6 CF Center, Università degli Studi di Milano.Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Via Commenda 9, 20122 Milan (Italy)

ABSTRACT

Objectives: there are good arguments to believe that faecal pH is indicative of pH in the small bowel. The aim of the present study was to assess possible associations of the coefficient of fat absorption (CFA) with faecal pH and free fatty acids (FFA) in faeces in a cohort of patients with CF who had followed for the first time an evidence-based method to adjust pancreatic enzyme replacement therapy (PERT).

Design: patients followed a 24h pilot study consisting of a fixed diet with corresponding fixed doses of PERT, according to the prediction of the optimal dose by means of an *in vitro* digestion methodology. Faeces corresponding to the study period were marked with dyes. Total fat and total FFA profile were determined. The CFA was calculated, and the pH of the samples was measured. Linear mixed regression models were applied to explain the results.

Results: the median age of the 43 patients included was 9.4y. Median faecal pH and CFA were 6.09 and 90% respectively and they were positively associated with each other ($p<0.001$). An inverse relationship was found for pH and total fat and total FFA ($p=0.048$). The faecal pH was associated with longer transit time ($p=0.05$) and the use of omeprazole ($p=0.009$). Oleic, stearic and palmitic acids were the predominant FFA found in faeces, what is in accordance to the dietary intake. Older patients presented the same profile of FFA characterised by low presence of the assessed spectrum ($p=0.013$).

Conclusion: The pH of the faeces is suggested as the reason for the differences found in the CFA in a cohort of patients with CF that followed the same diet and had the same doses of PERT according to an evidence-based method to optimally adjust it.

Keywords: cystic fibrosis, pancreatic insufficiency, digestion, pancreatic enzyme replacement therapy (PERT), coefficient of fat absorption (CFA), faeces, pH, free fatty acids

1. INTRODUCTION

Pancreatic insufficiency is present in around 85% of the patients with Cystic Fibrosis due to the dysfunction or absence of the CFTR protein in the pancreatic duct cells. The obstruction of the duct impairs that digestive pancreatic enzymes are poured to the intestine, resulting in nutrient maldigestion and malabsorption [1]. While carbohydrates and protein reach satisfactory hydrolysis, the digestion of fat is the most compromised [2,3], as lipase activity requires an alkaline pH for an optimal activity and in

CF the secretion of sodium bicarbonate poured into the duodenum is decreased [4,5]. When a triglyceride molecule is completely hydrolysed it results in the production of free fatty acids, which can be absorbed by the gut cells [6,7].

Maintaining an adequate nutritional status is a priority in the CF treatment as it has repeatedly proven to be associated with better disease prognosis and survival [8,9]. Pancreatic Enzyme Replacement Therapy (PERT) is therefore the indicated treatment to compensate for this situation and to prevent malnutrition [1,10]. However, establishing an optimal PERT dose is a difficult target in the clinical practice. Patients with similar characteristics may have different requirements of PERT dose, and the same patient needs different doses depending on the meals. In fact, the guidelines on nutrition in CF state there is a low degree of evidence in the current recommendation of 2000-4000 LU/g fat [1]. Several authors have hypothesised that both individual patients' characteristics and food properties may have an impact on the efficacy of the enzymatic supplements, this resulting in different dose requirements for different meals [11,12]. In fact, previous research confirmed that small bowel pH was determinant of the efficacy of PERT [13,14].

Responding to the need of an evidence based-method to adjust PERT, MyCyFAPP Project (www.mycyfapp.eu) was funded by the European Union to address this challenge [15]. In a first approach, an *in vitro* digestion model was set up to simulate the CF gastrointestinal conditions in lab and to test a selection of foods under different doses of PERT. Modelling of the results allowed for the prediction of a theoretical optimal dose for each food, i.e. the theoretical optimal dose of enzymes (TOD) [13]. In a second step, a pilot study with 43 CF patients was conducted, in which the foods tested *in vitro* were consumed in a 24h test diet with their corresponding theoretical optimal dose [16]. Participants collected faeces during the test period to evaluate the efficacy of the model. Results revealed that the *in vitro* model was representative of the real physiological digestive environment, as median CFA was 90% (clinical target) and it was not associated to relevant patients' characteristics such as mutation, age or body mass index, although it was significantly associated to transit time [16]. Therefore, we

concluded that the proposed method to adjust PERT based on food properties was accurate enough and that patient characteristics' could not explain that in a subgroup of the study population CFA could not reach the 90% clinical target.

In the light of this new finding, the hypothesis was raised that other clinical measurements should be taken into account to explain the differences in CFA among patients using the same amount of PERT for a same meal as was performed in our study. The focus of the hypothesis was placed on the intestinal pH.

Therefore, the primarily objective of the present study was to further investigate the association of CFA with the faecal pH (as a substitute of the small intestine pH). Secondarily we aimed to explore total FFA as complementary biological indicators of the result of fat digestion in the context of the application of an evidence-based method to adjust PERT in CF.

2. METHODS

2.1. Subjects and study design

The participating CF units, the inclusion and exclusion criteria and the study design are explain in detail in the previously publication by our group 16. Patients belonged to 5 European CF Units (Madrid, Valencia, Milan, Leuven and Rotterdam). To be eligible for the study they had to have a confirmed CF diagnosis, pancreatic insufficiency, age between 2 and 18 years old and a stable clinical status 2 weeks before signing the informed consent. The exclusion criteria contemplated the presence of acute infections, use of antibiotics, severe cholestasis and changes in the usual treatment 2 weeks before starting.

The study design consisted of the adherence to a 24h diet in which a fixed diet with a fixed PERT dose were prescribed according to the previously established method. During the study period faeces were collected as marked with colorimetric dyes in order to identify the faeces associated to the study period.

2.2. Faeces analysis

The faeces were shipped from all the participating centres to the central lab in Instituto de Investigacón Sanitaria La Fe (Valencia, Spain) for analysis. Homogenisation of samples was carefully carried out, only including the faeces corresponding to the test menu: the samples not showing red-dyed faeces were discarded. Then stirrers 750 W (Braun MQ735) were used to mix the faeces contained in the original recipients until complete homogenisation was observed (5 minutes per sample approx., depending on the consistency and volume). Careful rinsing of the equipment was performed between samples. None of the protocols for homogenisation referred in the literature were applied because the method was vaguely described or not available at all and also because there is no generally accepted standardised method 17-20.

Total fat in faces

Quantitative faecal fat analyses were carried out by the infrared spectroscopy technique, the golden standard to perform fat in faeces analyses at the Reference Laboratory (Barcelona, Spain). Random selected samples (n=10) were used to assess the homogenisation protocol developed. Test results were highly reproducible (CV <15%) between two different aliquots of the same homogenised sample.

Free fatty acids

Aliquots from the homogenised faeces samples (5 g) were liofilised and esterified. Then the FFA profile was characterised by means of gas chromatography – mass spectrometry (GC-MS) technique.

Coefficient of fat absorption (CFA)

The CFA was calculated as the percentage of grams of fat excreted in faeces (considering the faeces belonging to the test diet) compared to the grams of fat in the test diet. This parameter is considered equivalent to the % lipolysis extent used in the *in vitro* setting. The transit time was calculated as the time between the ingestion of the red capsule and the moment the first red stool appeared.

pH in faeces

Samples were thawed at room temperature. 5 g of faeces were mixed with 10 ml of deionized water and homogenized 1-2 min using, firstly, a vortex and then during 10min (200 rpm) using a horizontal shaker. Fecal pH was measured by directly inserting the glass electrode of the accumet AE150 pH Benchtop Meter (Fisher Scientific) into the homogenized feces. Measurements were taken twice from samples. In addition, pH was measured at three moments in a period of 24h and after a period of 1 month in order to assess possible changes in the measurement over time.

2.3. Statistical analysis

With the aim of studying the association of the faecal pH effect on the CFA, a beta regression model was carried out given the nature of the variable (ranging from 0 to 100). The association between faecal pH with the logarithmic transformation of the variable g fat in stools by means of a univariable linear regression model, and the effect of the faecal pH on total FFA (mg/g faeces= by means of a linear regression robust model. Finally, a linear regression multivariable robust model was established to study the association of the age, the logarithmic transformation of transit time and the use of PPI on the faecal pH.

Values of the individual FFA were represented for all the subjects by means of a heatmap allowing for the gathering per individual and per FFA which formed clusters based on the Euclidean distances matrix. In order to facilitate the visualization of the clusters the values of all the FFA were scaled. The scalation was centred in the mean value, so the colour scale represents the number of standard deviations that the value differs from the mean of the respective FFA.

Analysis were carried out using the R software (version 3.5.0) and the libraries betareg (version 3.1-0), clickR (version 0.3.64) and MASS (version 7.3-49). A p value <0.05 was considered statistitcally significant.

3. RESULTS

3.1. Patients characteristics and descriptive results

The study population characteristics was previously described by Calvo-Lerma et al. (2018). It consisted of 43 paediatric patients with CF with a median age of 9.4 years in follow up at 5 European CF centres. From the total sample 14/42 of the patients were supplemented with PPIs, and median transit time was found to be 23 (16.8, 40.6) h. Table 1 summarises these previous results along with the results of the analytical determinations performed on the collected samples of faeces.

Table 4.23. Clinical data of the patients and results of the analysis of the faeces samples

Parameter	Median value (1st, 3rd Q)
Total fat in faeces (g)	8.35 (4.85, 12.28)
Total FFA in faeces (mg/ g faces)	2.27 (1.55, 3.64)
CFA (%)	90 (84, 94)
pH in faeces	6.08 (5.78, 6.38)
Use of PPI	33% of the patients
Transit time (h)	23 (16.8, 40.55)

3.2. Total fat, total free fatty acids and pH in faeces

As shown in **Table 4.23**, the median total amount of fat in faeces was 8.35 (4.85, 12.28) g what lead to a median CFA of 90% with a small range between 1st and 3rd Q (84, 94%). The total amount of free fatty acids was 2.27 (1.55, 3.64) mg/g of faeces sample. The median pH of the samples was 6.08 (5.78, 6.38), with a minimum pH of 5.26 and a maximum of 6.85. The reproducibility of the measurement was high, with a mean standard deviation of 0.02 when measuring the pH at three different points of the homogenized sample. Changes in the pH measurement along a 24h period were not detected.

The pH of faeces had a statistically significant positive association with the CFA ($p<0.001$) (**Table 4.24**), so that higher pH values were related to higher CFA. This result was reinforced by the fact that there was a significant inverse relationship between the pH and the total amount of fat in faeces and the total amount of FFA ($p<0.001$ and $p=0.048$ respectively): the lower the pH the higher the total amount of fat and FFA in faeces.

The explanation for the resulting pH value in faeces was explored. A higher pH of faeces was significantly associated with a longer transit time ($p=0.05$) and with the use of PPI ($p=0.009$) in a multivariable context (**Table 4.25**). In contrast, the age of the patient did not show any effect on this result and neither any of the other study variables.

Table 4.24. Univariate linear regression models to explore the association between pH of faeces and the fat excretion parameters: total fat, total free fatty acids and CFA.

	Estimate	Std. Error	95% Confidence interval	P-value
Total fat (g)	-1	0.24	[-1.486, -0.514]	<0.001
Total FFA (mg/g faeces)	-0.931	0.491	[-2.247, -0.025]	0.048
CFA (%)	1.104	0.225	[1.041, 4.683]	<0.001

Table 4.25. Robust linear regression model explaining the association between age, transit time and use of proton pump inhibitors on the faces samples pH value

	Estimate	Std. Error	95% Confidence interval	P-value
Age (years)	0.017	0.014	[-0.02, 0.042]	0.245
Transit time (h)	0.254	0.128	[0.003, 0.567]	0.050

	Estimate	Std. Error	95% Confidence interval	P-value
Proton pump inhibitors (yes)	0.358	0.129	[0.07, 0.583]	0.009

3.3. Free fatty acids profile

The CFA did also show a significant association with the total FFA detected in faeces (p=0.031), so that the lower FFA, the higher CFA. **Table 4.26** presents the quantification of the individual free fatty acids as referred to the faeces sample and the total amount of fat. Palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:1) were found in the highest amounts as compared to the rest of the assessed fatty acids. In contrast, the fatty acids with the shorter chain, i.e. capric acid (C10:0) and myristic acid (C14:0), were found in close to 0 amounts, and the same result was obtained for very long chain fatty acids: arachidic acid (C20:0) and behenic acid (C22:0).

Table 4.26. Total amount of individual free fatty acids in faeces referred to faeces sample weight and to gram of undigested fat

	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C20:0	C22:0	Total
mg/ g faeces *Median (IQR)*	0 (0, 0.01)	0.16 (0.08, 0.24)	0.04 (0.02, 0.07)	0.54 (0.34, 0.79)	0.41 (0.24, 0.74)	0.68 (0.38, 1.28)	0.2 (0.12, 0.33)	0.03 (0, 0.12)	0.04 (0, 0.08)	2.27 (1.55, 3.64)
mg/ g fat *Median (IQR)*	0 (0, 0.04)	0.91 (0.6, 1.47)	0.23 (0.15, 0.34)	3.8 (2.31, 5.05)	3.03 (1.65, 3.9)	4.72 (2.62, 7.91)	1.39 (0.53, 2.49)	0.22 (0, 0.73)	0.27 (0, 0.58)	16.49 (10.11, 24.57)

C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C20:0, arachidonic acid; C22:0, behenic acid

In addition, the individual patients' free fatty acids were plotted by means of a heatmap (**Figure 4.27**) and the clusters of possible common patterns were depicted.

Approximately, more than a third of the sample was clustered with the same pattern (right part of the figure), this being characterised by the low presence of most of the FFA, especially C16:0, C18:0, C20:0 and C18:1. The heatmap also gathered the patients with an overall higher amount of free fatty acids (left side of the heatmap) showeing that oleic acid (C18:1), the majoritarian fat in the foods of the test diet, was in all of them in high amounts.

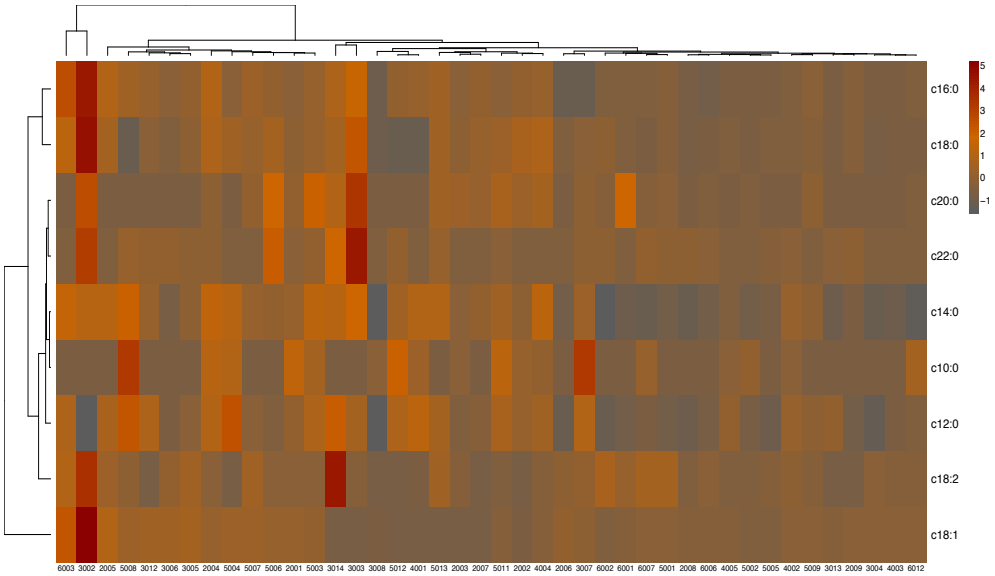


Figure 4.27. Heatmap displaying the free fatty acids profile in faeces in the participating centres. Columns represent the patients and rows the free fatty acids. The colour scale indicates the presence (the darker colour corresponding to a lower amount). *C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C20:0, arachidonic acid; C22:0, behenic acid*

Complementarily, the patients' characteristics determining the free fatty acid profile in faeces were explored (**Table 4.27**). The only reason for a series of patients presenting the same amount of a series of FFA was the age (p=0.013), and neither the transit time or the use of PPI were significantly associated to this result.

Table 4.27. Multivariate regression model explaining the effect of age, transit time and use of PPIs on the release of C16:0, C18:0, C20:0 and C18:1

	Estimate	Std. Error	95% Confidence interval	P-value
Age	-0.054	0.02	[-0.112, -0.014]	0.013
Transit time	-0.188	0.183	[-0.463, 0.351]	0.31
Use of PPIs	-0.175	0.182	[-0.527, 0.206]	0.344

4. DISCUSSION

Through the present study we have characterised total fat, total free fatty acids and pH of faeces samples belonging to a cohort of paediatric patients with cystic fibrosis who had followed for the first time an evidence-based method to adjust PERT, including the adherence to a fixed diet and fixed doses of PERT. First, we have found that FFA amount is associated to CFA, so that the higher the coefficient of fat absorption the lower the total FFA. This can be interpreted as that patients in which the fat digestion was high, the absorption of free fatty acids was high too, as low amounts remained in faeces. Thus, this finding suggests that when the TOD of PERT is achieved, fat is not only well digested but also well absorbed. Secondly, the faeces pH had a significant positive association with the CFA, plus had a significant inverse relationship with the total fat and the total free fatty acids. In our previous study we concluded that when using the TOD, CFA was not associated to any of the assessed patients' characteristics except from transit time. Additionally, the use of PPIs had not a significant impact on CFA, but described a positive tendency. We concluded that other patients' characteristics could have had an impact on CFA too, had these been possible to be assessed, i.e. the intestinal pH [16]. Thus, this new finding could explain why in our previous study some patients did not reach the 90% target CFA, confirming the hypothesis of the present work. Finally, the analysis of the free fatty acids profile in faeces showed there was a cluster of patients presenting a low amount of all the assessed components, and another presenting higher amounts, especially of oleic acid. This pattern was associated

with the age, so that the patients with the lower amounts of a series of free fatty acids were the oldest.

Our results are in accordance to the literature on related fields. A previous research conducted by our group showed the strong influence of the intestinal pH on lipolysis under simulated *in vitro* gastrointestinal digestion [13]. Besides, another study conducted with patients undertaking enteral nutrition allowed for the estimation of the intestinal pH, which was positively associated with the extent of fat digestion [14]. Given the impossibility to assess intestinal pH in our patients (ethical restrictions, invasive techniques…) we measured the pH in faeces as an indirect measurement of the intestinal pH. Possible explanations for the values of pH in faeces were explored, finding that patients using PPIs and with longer transit times had higher values of this measurement.

It is well known that the use of PPI has an inhibitory role of the gastric juice secretion, what makes the stomach environment, normally at around pH 3, less acidic during digestion [21]. As a consequence, the gastric content enters the duodenum with a more alkaline pH than the usual, thus modifying the intestinal conditions towards a normal environment, despite the limited pancreatic secretion of bicarbonate [15], and supporting the use of PPIs in CF cases with severe PI and difficult to control steatorrhea. Despite this therapy has proven to improve digestion and absorption of nutrients and nutritional status in patients with CF, the long term application may lead to complications [22-23].

We could not find any literature, however, specifically addressing the study of the transit time and its impact on the intestinal pH profile during digestion. Longer transit times associated to higher pH is coherent with a study conducted in rats fed with different amounts of fibre, which concluded that shortening the transit time suppresses fat absorption [24]. A possible explanation could be that when the transit time is longer, there progressive alkalinisation of the digestion medium is achieved to a higher extent than when digestion occurs faster.

Some studies exploring the pH profile along digestion in healthy population by using the wireless motility capsule technique showed that there is an increasing rate of the pH along the small intestinal digestion [25] up to a certain point when it decreases. After the colonic stage, the pH increases again reaching a similar profile as in the duodenum [25-27]. In the case of CF, the range of pH along the first hour of small intestine digestion oscillates between 5 and 7 [26,28] . Based on these studies, we found that the pH range found in the faeces samples of the present study are coherent, as they are in the same range as the intestinal pH of the subjects previously studied.

As for the free fatty acids profile in faeces, a previous study conducted with at term newborns and fed with an infant formula, did show a 10 times higher amounts of free fatty acids in faeces, this fact being related to the immaturity of the digestion system. In that study, the oleic (C18:1) and the palmitic acid (C16:0) were also those in the highest proportions [29]. We could not find any study specifically assessing the association between fat digestion extent and its products in faeces.

The main limitation of the present study is the assumption that the pH in faeces is the pH in the small intestine. In the colonic stage, several reactions may lead to the modification of the pH value the digestion contents had during the previous phases, including the fermentation of unabsorbed or undigested nutrients (carbohydrates, protein) by some specific bacteria conforming the microbiota. This results on the production of short chain fatty acids, which may reduce the luminal pH. Even if the study subjects could have different microbiota profiles, the production of the short chain fatty acids is highly dependent on the pattern of food intake [30], and all of our patients had the same meals. Another factor possibly modifying the pH at the colon is related to carbohydrates malabsorption [31], but there was not any reason to suspect carbohydrates malabsorption in our population, since normally it is associated to liquid depositions [32], which were rarely found in our population [16]. However, at the light of the significant association between the pH in faeces and the total fat in stools, total faecal free fatty acids and CFA, plus the comparable range of pH as in previous series, we consider this indirect measurement is robust and representative enough of the

intestinal conditions. Another limitation of this study is the scarce literature of similar studies conducted in the field, which has restricted the comparison of our results to other series.

In contrast, the strengths of this study include the accuracy of the collection of the faeces samples, since colorimetric dyes were used to identify those depositions belonging to the study period. The samples are of unique value since for the first time to our knowledge, they represent the product of the digestion of the same diet and the same PERT doses followed by a cohort of patients with CF. Thus, it has been possible to assess differences in the CFA only in terms of individual patients' characteristics, avoiding the major confounder factors related to the diet or the variability in the dose. Another strength relies on the GC-MS technique used, the most accurate method to quantify free fatty acids [33].

The strong association between faeces pH and the extent of fat digestion reinforces the previous conclusions of our group's research, that in the clinical practice we should apply those strategies that allow for increasing the intestinal pH value. Our results prove that the use of PPI lead to increased pH in faeces, and this increased pH in faeces was associated to more fat digested. Thus, our results support this co-adjuvant therapy to PERT should be applied in those patients with lower faecal pH to improve PERT efficiency, as its long term application may lead to complications. We suggest further strategies to be explored, including the encapsulating sodium bicarbonate by means of release-controlled coatings that would allow for the release in the duodenum.

Another clinical application, although with possible limitations, would be measuring pH in faeces as a complementary qualitative method to assess fat digestion. It is a simple and fast technique that only requires a pH-metre, a common and not expensive equipment. The measure is conducted by introducing the catheter into the homogenised sample and testing different areas of the sample. Besides the reproducibility of the measure resulted very high. This technique could complement the 24h faeces collection. In a free diet and free PERT dose setting, this would shed

additional information of the result of total fat in faeces, i.e. if this was due to an inadequate dose of PERT (high pH, high undigested fat) or to the intestinal pH of the patient (low pH, high undigested fat). However, this approach should be validated by conducting the study on a wider number of patients, and possibly using complementary analysis, such as measuring intestinal pH.

In conclusion, the pH of the faeces is suggested as the reason for the differences found in the CFA in a cohort of patients with CF that followed the same diet and had the same doses of PERT according to an evidence-based method to optimally adjust it. Thereby, strategies to increase the intestinal pH in CF are encouraged, including the use of PPIs and possible future therapeutic approaches, in order to enhance lipids digestion.

Acknowledgments

Authors of this paper acknowledge the support of the MyCyFAPP Project consortium. We especially thank the participation and the effort of the patients involved in the study and their families.

Financial support

This work was fully funded by the European Union and the Horizon 2020 Research and Innovation Framework Programme (PHC-26-2014 call Self management of health and disease: citizen engagement and mHealth) under grant number 643806.

Conflict of interests

All authors, after having read the journal's policy on conflicts of interest, declare that there are no competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

5. REFERENCES

1. Turck, D. *et al.* ESPEN-ESPGHAN-ECFS guidelines on nutrition care for infants, children, and adults with cystic fibrosis. *Clin. Nutr.* **PublishAh,** (2016).
2. Fieker, A., Philpott, J., & Armand, M. (2011). Enzyme replacement therapy for pancreatic insufficiency: present and future. Clinical and experimental gastroenterology, 4, 55.
3. Alpers D. Digestion and absorption of carbohydrates and proteins. In: LR J, editor. Physiology of the Gastrointestinal Tract. Vol. 2. New York: Raven Press; 1994. pp. 1723–1749.
4. Gelfond D, Ma C, Semler J, Borowitz D (2013) Intestinal ph and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. Dig Dis Sci 58:2275–81.
5. Robinson PJ, Smith AL, Sly PD, Al RET (1990) Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. Dig Dis Sci 35:1299–304.
6. Hunter, J. E. (2001). Studies on effects of dietary fatty acids as related to their position on triglycerides. Lipids, 36(7), 655-668.
7. Hernell O, Staggers JE, Carey MC. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. Biochemistry. 1990;29(8):2041–2056
8. Yen EH, Ms HQ, Borowitz D. Better Nutritional Status in Early Childhood Is Associated with Improved Clinical Outcomes and Survival in Patients with Cystic Fibrosis. *J Pediatr*. 2013;162(3):530-535
9. Corey M, Mclaughlin FJ, Williams M, Levkon H. A COMPARISON OF SURVIVAL , GROWTH , AND PULMONARY FUNCTION IN PATIENTS WITH CYSTIC FIBROSIS IN BOSTON AND TORONTO. *J Clin Epidemiol*. 1988;41(6):583-591
10. Borowitz D, Baker RD, Stallings V. Consensus report on nutrition for pediatric patients with cystic fibrosis. J Pediatr Gastroenterol Nutr 2002;35:246 – 59
11. Li, Y., Hu, M. & McClements, D. J. Factors affecting lipase digestibility of

emulsified lipids using an in vitro digestion model: proposal for a standardised pH-stat method. *Food Chem.* **126,** 498–505 (2011).

12. Schall, J., Bentley, T. & Stallings, V. Meal patterns, Dietary Fat Intake and pancreatic enzyme use in preadolescent children with Cystic Fibrosis. *J. Pediatr. Gastroenterol. Nutr.* **43,** 651–659 (2006)
13. Calvo-Lerma, J. *et al.* Evidence-based method to adjust Pancreatic Enzyme Replacement Therapy in Cystic Fibrosis: Part I, in-vitro study. *J. Cyst. fibrosisU* **Under revi,** (2018).
14. Robinson PJ, Smith AL, Sly PD, Al RET (1990) Duodenal pH in Cystic Fibrosis and Its Relationship to Fat Malabsorption. Dig Dis Sci 35:1299–304.
15. Calvo-Lerma, J. *et al.* Innovative approach for self-management and social welfare of children with cystic fibrosis in Europe: development, validation and implementation of an mHealth tool (MyCyFAPP). *Br. Med. J. Open* **7,** e014931 (2017).
16. Calvo-Lerma, J. *et al.* Evidence-based method to adjust Pancreatic Enzyme Replacement Therapy in Cystic Fibrosis: Part II, in-vivo validation of the in vitro model. *J. Cyst. Fibros.* **under revi,** (2018)
17. Koumantakls, G. & Radciltf, F. J. EstimatingFat in Feces by Near-Infrared ReflectanceSpectroscopy. **33,** 502–506 (1987).
18. Rivero-marcotegui, A., Olivera-olmedo, E., Palacios-sarrasqueta, M., Grijalba-uche, A. & Garcı, S. Water , fat , nitrogen , and sugar content in feces : reference intervals in children. **1544,** 1540–1544 (1998).
19. Korpi-steiner, N. L., Ward, J. N., Kumar, V. & Mcconnell, J. P. Clinica Chimica Acta Comparative analysis of fecal fat quantitation via nuclear magnetic resonance spectroscopy (1 H NMR) and gravimetry. *Clin. Chim. Acta* **400,** 33–36 (2009).
20. Dorsey, J. *et al.* Fat malabsorption in cystic fibrosis: comparison of quantitative fat assay and a novel assay using fecal lauric/behenic acid. *J. Pediatr. Gastroenterol. Nutr.* **50,** 441-446 (2010).
21. Kalantzi L, Goumas K, Kalioras V, Abrahamsson B, Dressman JB, Reppas C. (2006)

Characterization of the Human Upper Gastrointestinal Contents Under Conditions Simulating Bioavailability / Bioequivalence Studies 23:165-176.

22. Proesmans M, De Boeck K. (2003) Omeprazole, a proton pump inhibitor, improves residual steatorrhoea in cystic fibrosis patients treated with high dose pancreatic enzymes. Eur J Pediatr 162:760–3.
23. Tran TMD, Neucker A Van Den, Hendriks JJE, Forget P, Forget P. (1998) Effects of a proton-pump inhibitor in cystic fibrosis 87:553-8
24. Munakata A, Iwane S, Todate M, Nakaji S, Sugawara K. (1995) Effects of dietary fiber on gastrointestinal transit time, fecal properties and fat absorption in rats. J Exp Med 176:227-238
25. Fallingborg, J., Christensen, L. A., Ingeman-Nielsen, M., Jacobsen, B. A., Abildgaard, K., & Rasmussen, H. H. (1989). pH-profile and regional transit times of the normal gut measured by a radiotelemetry device. Alimentary pharmacology & therapeutics, 3(6), 605-614.
26. Fallingborg, J. (1999). Intraluminal pH of the human gastrointestinal tract. Danish medical bulletin, 46(3), 183-196.
27. Aburub A, Fischer M, Camilleri M, Semler JR, Fadda HM (2018). Comparison of pH and motility of the small intestine of healthy subjects and patients with symptomatic constipation using the wireless motility capsule. Int J Pharm, 544:158-164
28. Gelfond, D., Ma, C., Semler, J., & Borowitz, D. (2013). Intestinal pH and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. Digestive diseases and sciences, 58(8), 2275-2281
29. Lopez-Lopez, A., Castellote-Bargallo, A. I., Campoy-Folgoso, C., Rivero-Urgel, M., Tormo-Carnice, R., Infante-Pina, D., & López-Sabater, M. C. (2001). The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn faeces. Early human development, 65, S83-S94.
30. Fiorentini, G., Carvalho, I. P., Messana, J. D., Canesin, R. C., Castagnino, P. S.,

Lage, J. F., ... & Berchielli, T. T. (2015). Effect of lipid sources with different fatty acid profiles on intake, nutrient digestion and ruminal fermentation of feedlot nellore steers. Asian-Australasian journal of animal sciences, 28(11), 1583.

31. Perman, J. A., Modler, S., & Olson, A. C. (1981). Role of pH in production of hydrogen from carbohydrates by colonic bacterial flora. Studies in vivo and in vitro. The Journal of clinical investigation, 67(3), 643-650.

32. Sellin, J. H., & Hart, R. (1992). Glucose malabsorption associated with rapid intestinal transit. American Journal of Gastroenterology, 87(5).

33. Zelles, L., & Bai, Q. Y. (1993). Fractionation of fatty acids derived from soil lipids by solid phase extraction and their quantitative analysis by GC-MS. Soil Biology and Biochemistry, 25(4), 495-507

5. SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

5.1. SUMMARY OF THE RESULTS

Before presenting the conclusions of this thesis, a brief summary of the results is provided as a means of recapitulation.

The first stage of the research, which examined the current cystic fibrosis nutritional management in European countries, showed that very different PERT dosing criteria were being applied in the cystic fibrosis units regardless of the medical guidelines recommendations. What is more, in all the centres a wide intra-patient variability in the administrated dose along eating events was detected. In addition, despite different country-specific dietary patterns that were found, in all of them high intakes of sugar and saturated fatty acids were a common outcome, along with a low consumption of fish, legumes, nuts, fruit and vegetables and a high consumption of processed foods.

The second stage explored the food properties and the gastrointestinal conditions and their impact on lipolysis. The *in vitro* digestion studies conducted in real foods shed light on the understanding of the mechanisms underpinning lipolysis and PERT action. The intestinal pH, the bile salts concentration and the lipid concentration in the digestion medium were highly determinants of lipolysis kinetics and extent. Especially, the simulated normal digestion conditions of intestinal pH 7 and bile concentration 10 mM led to 30/52 assessed foods led to a lipolysis extent > 90% lipolysis extent, while in the CF-like scenario (pH 6, bile 1mM) this extent was only found in 1/52. A strong nutrient interaction, negatively affecting lipolysis, was also found in foods with low content of fat and high contents of protein or starch. As for the lipid structure within the food matrix, the oil-in-water emulsion allowed for the highest lipolysis extents under the CF-simulated digestion conditions as compared to the other structures, but it had not a significant effect when applying normal digestion conditions. In addition, those food matrices presenting low hardness conferred higher

viscosity to the digestion medium, what made lipolysis extent decrease. Finally, the food structure in terms of nutrient composition and texture, led to different profiles of free fatty acids released during digestion.

Overall, fat content of foods was not the only factor determining lipolysis, being food properties significant contributors to dietary fats digestion fates. In fact, PERT dosing in terms of lipase units per gram of fat, showed that increasing doses did not always lead to higher lipolysis extents. In the third stage of the research, when testing a selection of meals with different PERT doses, the modelling of the results allowed for obtaining predicting equations of the optimal dose according to food characteristics. With these models, a theoretical optimal PERT dose (TOD) was assigned to the test meals. Patients followed a pilot study in which these test meals were consumed together with the TOD, and faeces (marked with colours to delimit the study period) were collected in order to assess the amount of fat excreted and to calculate the coefficient of fat absorption. The results of the pilot study showed that when applying the TOD, median CFA was 90%, which is the clinical target, indicating that the PERT dose is optimally adjusted. Furthermore, patients' individual characteristics such as age, type of mutation and nutritional status did not have a significant effect on the CFA outcome. Complementarily, a more in detail study of the faeces, showed that there was a significant association between faeces pH (an indirect measurement of the intestinal pH profile) and CFA, confirming the same effect showed in the *in vitro* studies (higher intestinal pH, higher lipolysis). The final result confirmed that lower total excreted fat in faeces was associated with lower free fatty acids, what means that TOD not only led to high digestion of fat, but also to high absorption of its products.

5.2. CONCLUSIONS

1. The need for an evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis has been reaffirmed: there is a wide intra-centre and intra-patient dosing range being applied in Europe.

2. The dietary intervention in cystic fibrosis should be focused on promoting the consumption of healthy fats, such as fish and nuts, in order to revert the current situation in which processed foods are main source of this nutrient: the increasing life expectancy in cystic fibrosis claims for the adherence to healthy diets in order to prevent nutrition-related disorders and complications.

3. High fat meals recommendation was reinforced for the following reasons: some patients do not reach the daily recommendation of fat intake; in foods with low fat content and high content of protein or starch lipolysis is decreased; and fat concentration in the digestion medium favours lipolysis extent.

4. The recommendation of PERT dose should not be based on fat content of meals anymore, as in addition food properties contribute significantly to the digestion of dietary lipids. A more complex dosing criterion is needed in order to optimise lipid digestion.

5. Gastrointestinal conditions, especially high intestinal pH can significantly increase lipolysis extent during digestion. Adjuvant therapies to PERT should be considered and applied in the clinical practice, and new strategies to increase intestinal pH should be further explored.

6. For the first time, an evidence-based method to adjust PERT was established, consisting of a theoretical optimal dose of enzymes based on food properties.

7. The PERT optimal theoretical doses as established by the *in vitro* model was applied in a pilot clinical trial, leading to the clinical target CFA, and can be therefore considered clinically validated in this context and used as the first evidence-based method to adjust pancreatic enzyme replacement therapy in cystic fibrosis.

6. FUTURE PERSPECTIVES

6. FUTURE PERSPECTIVES

The results of the research conforming this thesis could have an impact on the field of food science and technology and a potential application in the cystic fibrosis treatment clinical practice. Some of the results have been already used in the development of some of the products of MyCyFAPP Project, whose future exploitation is currently being explored.

6.1. GENERATED BASIC KNOWLEDGE IN THE FIELD OF FOOD SCIENCE

Most of the studies in the literature about nutrient digestion have been conducted on the basis of model systems, very few of them having addressed the complexity of the real food matrix.

The *in vitro* digestion method developed in the frame of this thesis has allowed for the exploration of lipolysis of a wide range of foods, belonging to different food groups, implying different nutritional compositions and physicochemical properties. This endeavour clearly supposes a step beyond in the state of the art. The generated knowledge may be of interest in future studies addressing the lipolysis in real foods, and even the methodology could be reproduced for similar research purposes.

From a more practical outlook, unveiling of the role of the nutrient interactions and the lipid structure within the food matrix can be of use in the field of new and functional foods development. Food structure for food design is a growing tendency, which mainly aims at modulating nutrient digestion and absorption for certain purposes, such as the prevention and treatment of obesity by means of several strategies, based on food matrix properties.

6.2. CLINICAL APPLICATIONS

Up to now, recommendations of food intake in Cystic Fibrosis have been made on the premise of achieving high-fat diets. However, we have proved that different foods, according to their properties such as nutrient composition and structure, lead to different fat digestion fates, independently of their content of fat. What is more, this finding was of special relevance when the simulated digestion was made with the Cystic Fibrosis intestinal conditions. Therefore, despite supposing a complex challenge, this new knowledge, should be applied in order to make new dietary recommendations for patients with Cystic Fibrosis. These recommendations should be based on the ability of PERT to digest fat, prioritising the intake of those foods achieving high lipolysis extents under the unfavourable intestinal digestion conditions characteristic of Cystic Fibrosis.

In addition, the identification of the optimal dose of enzymes for specific foods should be also considered when establishing dietary recommendations. The combination of different foods in a meal may lead to an optimal predicted dose that is excessively high, and therefore recommending splitting some of the foods conforming the meal into different eating events could be an effective strategy to maximise fat uptake while not supposing a high dose of the supplement.

Complementarily, a conclusion of this research suggests that lipolysis kinetics and extent can highly benefit from improving the conditions of the intestinal digestion medium. Thus, current therapies that have proved to increase the intestinal pH profile, i.e. proton pump inhibitors, could be applied in the clinical practice in those cases in which fat digestion is a challenging target. Furthermore, new possible adjuvant therapies to PERT should be explored. Possible strategies would include the encapsulation of sodium bicarbonate and/or bile salts by means of pH sensitive coating encapsulation systems that would revert the unfavourable CF intestinal conditions and likely favour the action of PERT, what would enable for a more efficient lipid digestion.

6.3. MyCyFAPP PROJECT

The study of the nutritional habits allowed for the identification of current imbalances present in the diet of European children with CF. This information was used as the basis to develop a nutritional recommendations handbook addressed to patients with CF and their families, aimed at correcting them and achieving the nutritional goals established in the clinical guidelines. The handbook is being published for the use of the patients. In addition, its contents have been adapted to the electronic format and has been added to the self-management app, including interactive features that smooth the exploration of its contents.

The other tools developed in the context of the European study on nutritional habits are also of potential use in future research. The specifically developed food record template along with MyFoodREC online system and MyFoodFACTS nutritional composition database – including food groups - can be used in nutritional habits studies, allowing for the reliable comparison of populations from different countries.

The evidence-based method to adjust PERT, based on the results conducted in the frame of the *in vitro* digestion studies, has been clinically validated in the framework of the pilot study. The methodology we have developed to explore the optimal dose of enzymes for the test meals was applied to a wide range of food products, and as a result, a theoretical optimal dose database has been built up. This database is the key feature of an optimal dose predictive algorithm that has been included in the self-management app, which allows patients with CF for the real-time automatic calculation of the optimal dose of enzymes for every possible meal. Currently, this feature along with the rest of the components of the app, is being tested in a clinical trial setting, in which 200 patients have been recruited, and whose results will see the light by the end of 2018.

Finally, the explained outcome products have been identified as exploitable by the MyCyFAPP consortium. At the moment intellectual property rights protection actions are being conducted, and patent registration is ongoing.

ANNEXES

ANNEX 1: Food record template used in the multicentre European survey on cystic fibrosis nutritional habits

LOGO OF THE HOSPITAL

MyCyFAPP

4-DAY FOOD RECORD

PAEDIATRIC CYSTIC FIBROSIS UNIT

MyCyFAPP Project

NAME:

DATE OF BIRTH:

How to Fill in the Food Record?

Dear father/mother/legal representative,

1. Fill in all the consumed foods during the next four days. Please detail:

a) **Name of the dish or foods** (e.g. macaroni)

b) **Ingredients** (e.g. pasta, mince, fried tomato, cheese, olive oil)

c) **Amount expressed as weight (grams) or approximated measure (household measures)** (e.g. 2 table spoons of macaroni, 150g of mince, 2 table spoons of fried tomato, 1 table spoon of olive oil)*.

d) **Preparation** (e.g. boiled, fried, breaded, baked…).

* In the following page you will find a table to help you to estimate the amounts of foods, both in grams and in household measures.

2. Do not forget to indicate sauces, seasonings, etc. that accompany the main course. Write the weight of it or an estimation of the consumed amount (e.g. five tomato slices, a small fried potato, two table spoons of grated cheese, etc.).

3. Indicate always if olive oil has been used in the preparation of the dish/meal, especially in pasta dishes, fired food, salads, sandwiches, etc.

4. Provide detailed information (name of the product and labeling if possible) in the case of pre-cooked meals (e.g. pizzas, nuggets, breaded fish, etc.) or manufactured products (pastries, chocolate biscuits, cereal bars, all kind of snacks, etc.).

5. in the 4th column:

- "CREON": write the amount (mg) supplied for each meal.

Note: Do not forget to include the name and the amount of the nutritional supplements (Fortimel®, Resource®, Protifar®, MCT Oil® or others) in your food record.

Table 1: Household measures

Household measure		Quantity
Spoon	Coffee	3g
	Tea	5g
	Soup	10-15g
Cup	Coffee	50ml
	Milk	150-200ml
	Cereals	250-300g
Glass	Small	50ml
	Medium	100ml
	Large	200ml
Plate	Desert	75-100g
	Shallow	150-150g
	Soup	200-300g

DAY 1 DATE:
NAME:

		DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
BREAKFAST	TIME:				Ursofalk®	
					Domperidone	
					Omeprazol	
					Vitamins	
					Nutrit. Suppl	

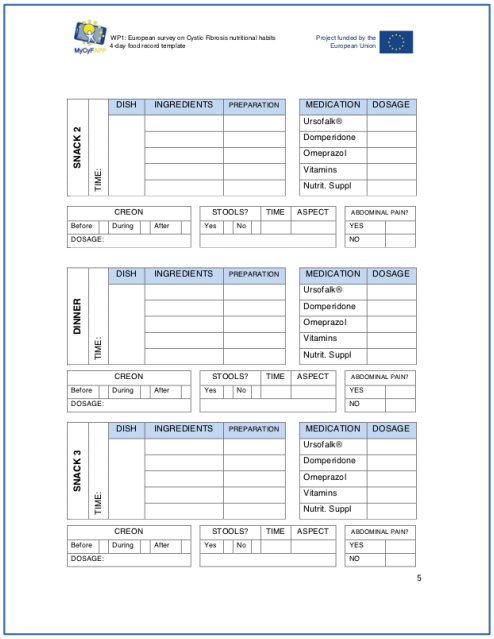
CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

		DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
SNACK 1	TIME:				Ursofalk®	
					Domperidone	
					Omeprazol	
					Vitamins	
					Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

		DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
LUNCH	TIME:				Ursofalk®	
					Domperidone	
					Omeprazol	
					Vitamins	
					Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
SNACK 2 TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

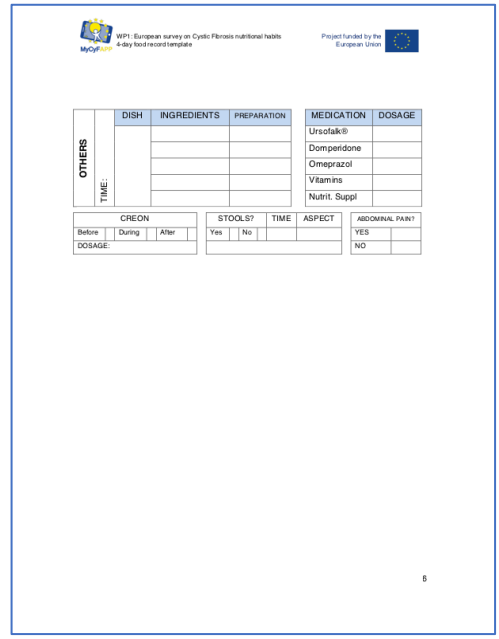
	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
DINNER TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
SNACK 3 TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

5



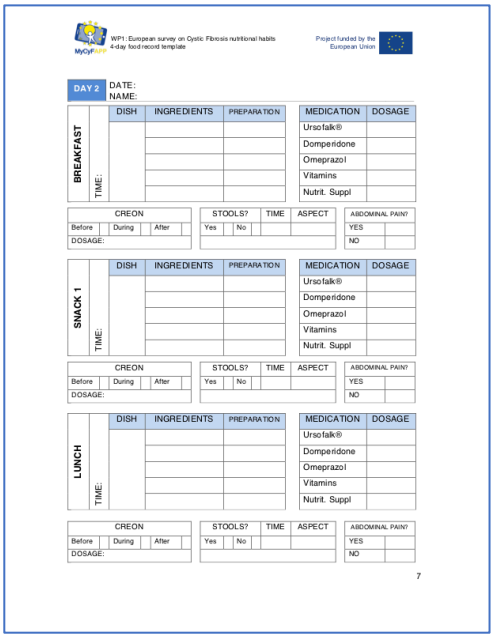
WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
OTHERS TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

6



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

DAY 2 DATE:
NAME:

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
BREAKFAST TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

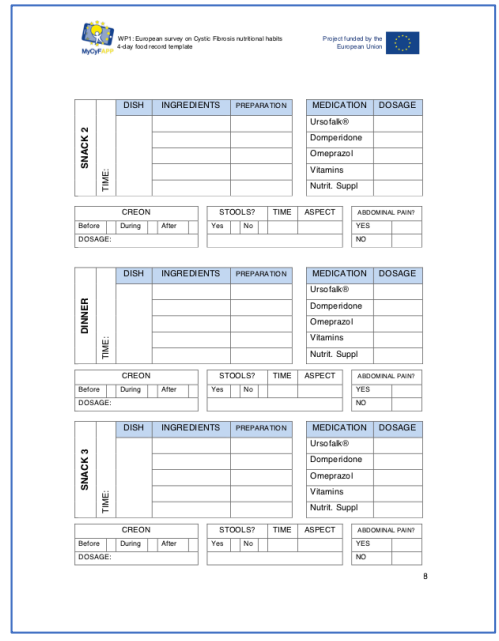
	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
SNACK 1 TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
LUNCH TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

7



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
SNACK 2 TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

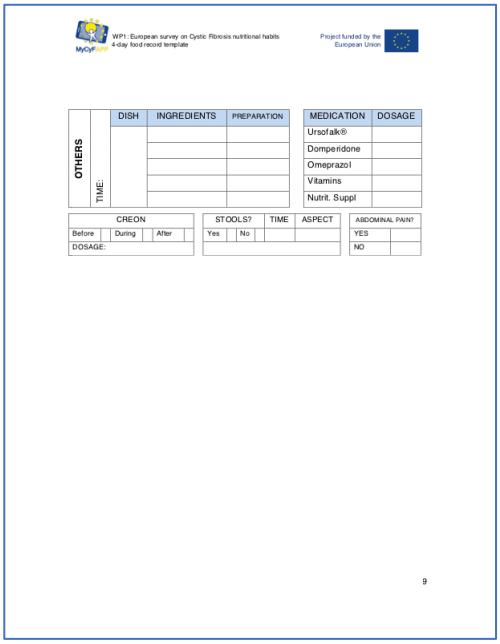
	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
DINNER TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

	DISH	INGREDIENTS	PREPARATION	MEDICATION	DOSAGE
SNACK 3 TIME:				Ursofalk®	
				Domperidone	
				Omeprazol	
				Vitamins	
				Nutrit. Suppl	

CREON			STOOLS?		TIME	ASPECT	ABDOMINAL PAIN?	
Before	During	After	Yes	No			YES	
DOSAGE:							NO	

8



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

OTHERS — TIME:

DISH	INGREDIENTS	PREPARATION

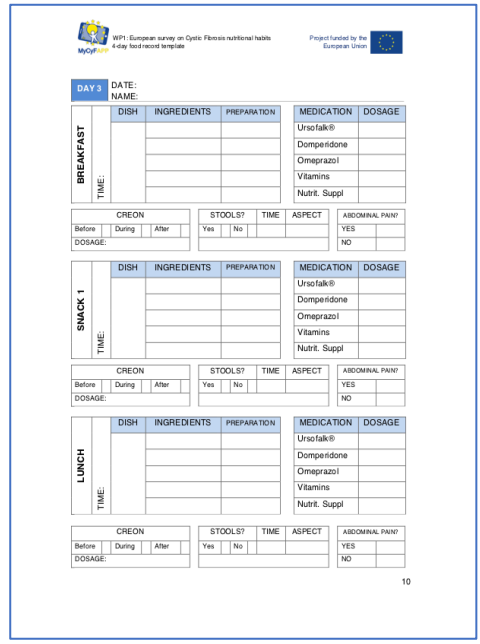
MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

9



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

DAY 3 DATE:
NAME:

BREAKFAST — TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

SNACK 1 — TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

LUNCH — TIME:

DISH	INGREDIENTS	PREPARATION

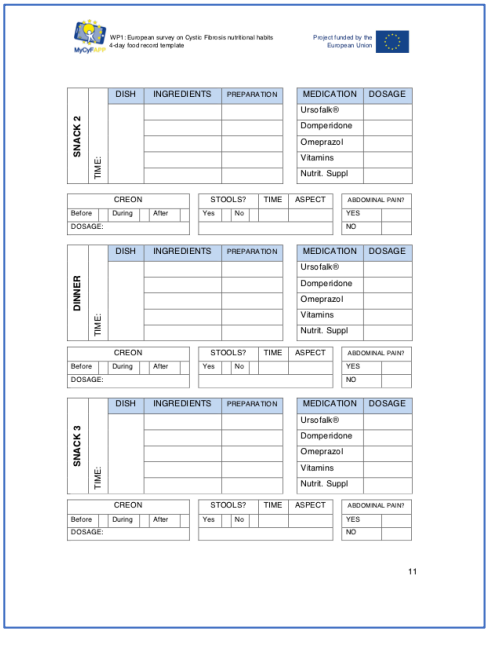
MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

10



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

SNACK 2 — TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

DINNER — TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

SNACK 3 — TIME:

DISH	INGREDIENTS	PREPARATION

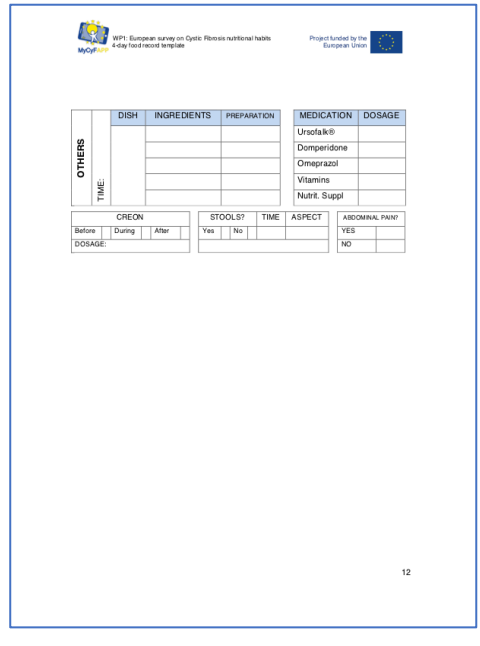
MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

11



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

OTHERS — TIME:

DISH	INGREDIENTS	PREPARATION

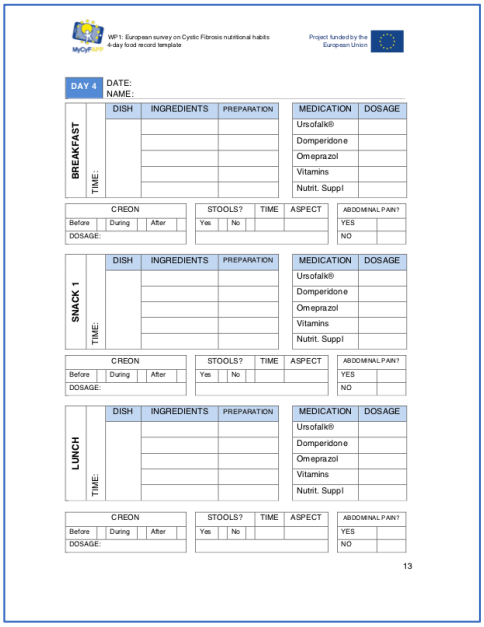
MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

12



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

DAY 4 DATE:
NAME:

BREAKFAST TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

SNACK 1 TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

LUNCH TIME:

DISH	INGREDIENTS	PREPARATION

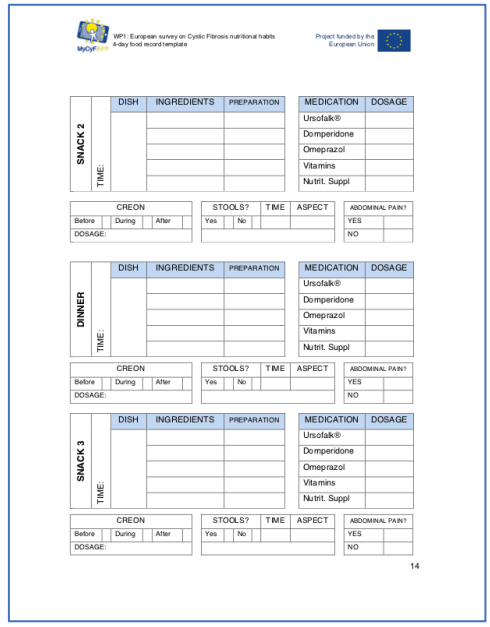
MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

13



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

SNACK 2 TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

DINNER TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

SNACK 3 TIME:

DISH	INGREDIENTS	PREPARATION

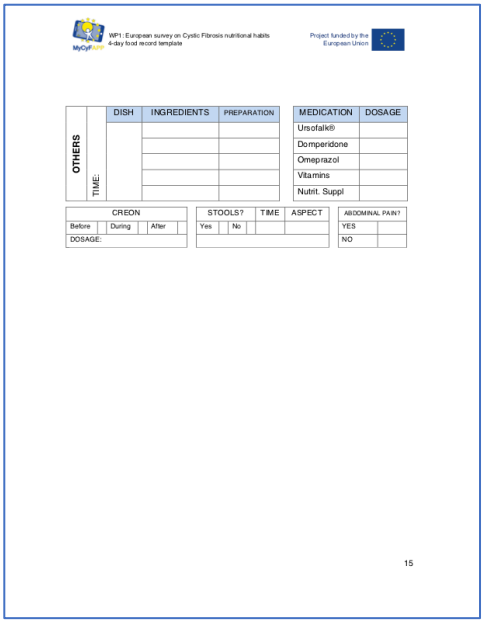
MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

14



WP1: European survey on Cystic Fibrosis nutritional habits
4-day food record template

Project funded by the European Union

OTHERS TIME:

DISH	INGREDIENTS	PREPARATION

MEDICATION	DOSAGE
Ursofalk®	
Domperidone	
Omeprazol	
Vitamins	
Nutrit. Suppl	

CREON		
Before	During	After
DOSAGE:		

STOOLS?		TIME	ASPECT
Yes	No		

ABDOMINAL PAIN?	
YES	
NO	

15

ANNEX 2: Formulation of the four bile compositions and concentrations

		Bovine	Porcine	Tauro-cholic (TC)	Tauro-cheno-deoxicholic (TCDC)	Glyco-cholic (GC)	Glyco-cheno-deoxicholic (GCDC)
F1	% (w/w)	100	-	-	-	-	-
	g/mol	440	-	-	-	-	-
F2	% (w/w)	-	100	-	-	-	-
	g/mol	-	440	-	-	-	-
F3	% (w/w)	50	-	5	5	20	20
	g/mol	220	-	26,85	24,98	97,4	94,32
F4	% (w/w)	50	-	20	20	5	5
	g/mol	220	-	107,4	99,94	24,35	23,58

w/w, weight/weight; F1, formula 1, bovine bile; F2, formula 2, porcine bile; F3, formula 3, high-glycocholic bile; F4, formula 4, high-taurocholic bile

ANNEX 3: Composition of simulated digestion fluids. The addition of pepsin, Ca2+ solution and water will result in the correct electrolyte concentration in the final digestion mixture

Constituent	SSF mmol · L^{-1}	SGF mmol · L^{-1}	SIF mmol · L^{-1}
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2$	1.5	0.15	0.6

Ca^{2+}, calcium ion; SSF, simulated salivary fluid; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; mmol, milimol; L, litre

ANNEX 4: Characteristic parameters of the log-logistic dose-response models for explaining kinetics in the three sets of experiments

Experiment	Conditions	Parameter *d*	CI 95%	Parameter *e*	CI 95%
a) Model 1. Gastric and Intestinal pH	pH 3 – pH7	92.12	[91.64, 92.6]	10.22	[10.07, 10.35]
	pH 3 – pH6	43.28	[41.62, 44.93]	30.23	[27.43, 33.01]
	pH 4 – pH7	99.03	[98.41, 99.64]	10.34	[10.16, 10.5]
	pH 4 – pH6	49.54	[47.93, 51.51]	34.29	[31.43, 37.13]
	pH 5 – pH7	102.15	[101.5, 102.8]	12.81	[12.61, 13.01]
	pH 5 – pH6	38.62	[37.83, 39.41]	37.53	[36.18, 38.87]
b) Model 2. Bile composition and concentration	F1 1mM	43.28	[41.62, 44.93]	30.23	[27.43, 33.01]
	F1 10mM	53.77	[52.57, 54.97]	26.42	[25.11, 27.72]
	F2 1mM	41.69	[40.92, 42.47]	27.022	[26.03, 28]
	F2 10mM	66.03	[63.35, 68.69]	51.55	[45.77, 57.31]
	F3 1mM	53.66	[50.17, 57.15]	34.03	[26.27, 39.79]
	F4 1mM	39.69	[38.49, 40.88]	36.56	[34.25, 38.86]

Experiment	Conditions	Parameter *d*	CI 95%	Parameter *e*	CI 95%
c) Model 3. Food fat content and ratio food/digestive fluids	LFF 0.5/1	39.22	[38.43, 40]	32.4	[31.18, 33.62]
	HFF 0.5/1	79.85	[49.65, 110.1]	70.80	[9.62, 131.98]
	LFF 1/1	43.28	[41.62, 44.93]	30.23	[27.43, 33.02]
	HFF 1/1	125.23	[98.2, 152.2]	93.06	[29.75, 156.4]
	LFF 2/1	47.87	[45.07, 50.68]	60.27	[53.61, 66.91]
	HFF 2/1	114.11	[99.22, 129]	85.39	[54.37, 116.4]

F1, formula 1, bovine bile; F2, formula 2, porcine bile; F3, formula 3, high-glycocholic bile; F4, formula 4, high-taurocholic bile; mM, milimolar; LFF, low fat food (5.5%); HFF, high fat food (35%)

ANNEX 5: Classification of foods according to the lipid structure within the food matrix

Food group	Food	Lipid structure	Lipid substructure	Ca2 (mg/	CH (g/10	Energy (k	MUFA (g/	PUFA (g/	SFA (g/1(	Fat (g/10	Fibre (g/	Na (mg/1	Protein (g	Starch (g	Sugar (g/	Fe (mg/1(
Dairy	Full Fat Milk	continuous aqueous phase	oil-in-water emulsion	117,0	4,6	63,0	1,1	0,1	2,4	3,6	0,0	45,0	3,1	0,0	4,6	0,1
	Semiskimed Milk			117,0	4,6	45,9	0,5	0,0	1,0	1,6	0,0	46,0	3,2	0,0	4,6	0,1
	Skim goat Milk			120,0	4,4	64,5	0,9	0,1	2,5	3,7	0,0	45,0	3,4	0,0	4,4	0,1
	Milk shake Choc			114,0	10,3	74,0	0,6	0,1	1,4	2,2	0,8	49,0	2,9	0,0	10,3	0,3
	Natural Yogurt			137,0	4,0	58,4	0,8	0,1	1,8	3,0	0,0	85,0	3,2	0,0	4,0	0,1
	Sugar yoghurt			119,0	12,9	84,0	0,6	0,0	1,2	1,9	0,0	92,0	3,0	0,0	12,9	0,2
	Liquid Yogurt			115,0	12,8	75,0	0,4	0,0	0,9	1,4	0,0	43,0	2,8	0,0	12,8	0,1
	Greek Yogurt			128,0	11,2	147,7	2,5	0,3	6,8	10,2	0,0	45,0	3,4	0,0	11,2	0,0
	Custard			129,0	17,2	106,9	0,8	0,1	1,8	2,8	0,0	67,0	3,4	2,8	14,4	0,4
	Creme caramel			91,0	20,4	131,0	1,8	0,5	2,0	3,4	0,2	63,0	4,8	0,0	20,4	0,7
	Petit Suisse			120,0	14,4	121,0	0,1	0,1	2,5	3,8	1,5	36,0	6,4	0,0	14,4	0,1
	Fresh cow cheese	complex solid structure	lipid inclusion in protein matrix	338,0	2,5	198,0	4,3	0,7	9,5	15,4	0,0	272,0	10,0	0,0	2,5	0,5
	Fresh goat cheese			543,0	1,1	296,8	6,2	0,8	15,4	23,9	0,0	480,0	19,8	0,0	1,1	0,4
	Cream cheese			200,0	3,0	240,0	7,4	0,7	15,0	23,0	0,2	460,0	5,0	0,0	2,5	0,3
	Soft cheese			470,0	1,6	384,0	9,2	0,9	17,2	32,0	0,0	670,0	22,5	0,0	0,0	0,9
	Cured cheese			767,0	0,0	407,0	9,9	1,0	23,1	34,0	0,0	670,0	24,0	0,0	0,0	0,6
Meat	Frankfurt Sausage	complex solid structure	lipid inclusion in protein matrix	20,0	1,3	290,0	12,0	3,0	9,3	26,0	0,1	900,0	12,7	0,7	0,6	1,0
	Pate			15,0	2,4	325,0	13,0	3,3	11,0	28,6	0,0	710,0	14,3	0,9	1,5	5,7
	Mixed Hamburguer			14,0	1,8	367,0	10,0	0,5	10,7	24,7	0,0	694,0	22,5	0,0	1,8	1,8
	Chopped Pork			14,0	1,4	323,0	13,3	3,0	10,8	29,0	0,0	1000,0	14,0	1,4	0,0	2,3
	Ham	continuous aqueous phase	intracellular lipid droplets and tissues	9,0	0,2	320,0	11,6	2,6	7,9	22,6	0,0	2130,0	28,8	0,0	0,2	1,7
	Cured ham			7,0	0,6	106,0	1,4	0,5	1,1	3,2	0,0	809,0	18,7	0,0	0,6	1,0
	Pork Loin			9,0	0,0	152,0	4,0	1,2	3,3	8,9	0,0	63,0	18,0	0,0	0,0	0,9
	Beef Steak			7,0	0,0	111,4	1,1	0,3	1,0	3,3	0,0	61,0	20,2	0,0	0,0	1,4
	Chicken drumstick			12,0	0,0	112,0	1,8	0,9	1,6	4,4	0,0	76,0	17,9	0,0	0,0	1,5
Fish	Salmon	continuous aqueous phase	intracellular lipid droplets and tissues	20,0	0,0	175,0	4,0	3,3	1,9	10,6	0,0	47,0	20,0	0,0	0,0	0,6
	Tuna			16,0	0,0	118,0	0,7	1,3	1,0	3,3	0,0	47,0	22,0	0,0	0,0	1,3
	Battered Hake	complex solid structure	lipid inclusion in CH and protein matrix	20,0	15,3	211,0	3,7	3,8	2,0	9,9	0,7	380,0	15,3	15,3	0,0	0,9
Egg	Poached egg	continuous aqueous phase	intracellular lipid droplets and tissues	56,0	0,4	150,0	4,4	1,7	2,8	10,8	0,0	133,0	12,6	0,0	0,4	2,0
	Hard egg			56,0	0,4	150,0	4,4	1,7	2,8	10,8	0,0	133,0	12,6	0,0	0,4	2,0
	Omelette			56,0	0,4	150,0	4,4	1,7	2,8	10,8	0,0	133,0	12,6	0,0	0,4	2,0
Nuts	Peanuts	continuous aqueous phase	intracellular lipid droplets and tissues	60,0	7,2	571,0	23,5	14,0	9,2	49,0	8,2	9,0	25,3	5,0	2,2	2,4
	Fried peanuts			48,0	8,9	590,6	24,2	14,4	9,5	50,3	8,4	320,0	26,8	4,0	4,9	1,7
	Walnuts			93,0	3,3	645,0	11,5	43,8	5,7	63,8	5,9	7,0	14,5	2,0	1,3	2,5
Fruit	Avocado	continuous aqueous phase	intracellular lipid droplets and tissues	16,0	0,8	138,0	8,9	1,8	2,9	14,2	3,0	7,0	1,8	0,0	0,8	1,0
Oils and fats	Olive oil	continuous lipid phase	free fat	0,0	0,0	889,0	73,3	8,9	12,8	99,9	0,0	0,0	0,0	0,0	0,0	0,0
	Butter		water-in-oil emulsion	15,0	0,4	742,0	24,9	2,6	50,9	83,0	0,0	22,0	0,6	0,0	0,4	0,2
Potato	Chips	continuous lipid phase	free fat	37,0	48,5	534,0	14,5	11,1	7,7	34,1	4,3	700,0	6,2	47,8	0,7	2,0
	French Fries			37,0	30,0	289,0	3,2	8,8	1,7	17,1	3,0	700,0	3,8	30,0	0,0	2,0
Sweets	Regular biscuit	complex solid structure	lipid inclusion in CH and protein matrix	118,0	76,3	436,0	3,5	1,5	5,7	11,5	2,6	217,0	6,7	52,5	23,8	2,0
	Choco-chip biscuit			78,0	64,3	489,6	9,0	6,0	7,0	22,9	1,8	220,0	6,2	37,6	26,7	1,3
	Choco breakfast cereal			25,0	87,0	534,0	1,2	4,7	5,4	1,5	5,4	700,0	4,5	35,8	36,2	6,5
	Crunchy biscuit bar			106,0	63,0	498,0	11,2	0,6	11,6	24,5	1,3	125,0	6,5	14,3	48,7	1,2
	Chocolate cake			25,0	39,6	381,0	6,8	1,0	13,1	21,9	0,8	46,0	6,5	16,1	23,5	0,9
	Muffin			25,0	39,9	385,0	8,1	0,9	12,4	22,4	1,0	211,0	6,1	20,7	19,2	1,1
	Croissant			42,0	55,0	405,0	5,5	0,8	9,9	17,2	2,2	492,0	7,5	47,5	7,5	1,2
	Donut			35,0	42,0	412,0	9,8	1,7	11,3	24,4	4,8	225,0	6,0	27,7	14,3	1,6
	Waffle			26,0	53,7	493,0	12,8	2,7	11,8	27,3	0,9	297,0	7,6	17,3	36,4	0,6
	Spreadable chocolate	continuous lipid phase	particles in solid fat	74,0	56,0	543,0	16,8	4,6	10,1	33,0	1,1	94,0	5,4	1,3	54,7	1,2
	Milk Chocolate			165,0	58,0	475,0	10,1	1,0	19,5	31,1	1,9	67,0	6,5	2,7	55,3	0,5
Cereal	Dark Bread	complex solid structure	lipid inclusion in CH and protein matrix	43,0	48,5	270,4	1,6	1,7	0,7	4,4	4,0	23,4	8,4	40,9	7,6	3,2
	White Bread			40,0	52,0	265,0	0,3	0,3	0,3	1,5	2,5	650,0	9,5	50,0	2,0	1,6

ANNEX 6: Protocol for the participant used in the pilot study

COVER PAGE

MyCyFAPP

MyCyFAPP: Innovative approach for self-management and social welfare of Cystic Fibrosis patients in Europe: development, validation and implementation of a telematics tool.

Funded by the European Union

Pilot study: development of the predictive model for the enzymatic optimal dose

STUDY PROTOCOL for the participant

PARTICIPATING CODE: _____

WELCOME

Dear participant in MyCyFAPP Pilot Study,

First of all we would like to express our gratitude in the name of the MyCyFAPP Project for your participation in the present pilot study. The pilot study is aimed at working out a predictive model of the optimal enzymatic supplements dose according to the meal characteristics and the patients' individual conditions. Investigators of the Project believe the results achieved, derived from your valuable contribution, shall suppose a great advance in the field of the pancreatic enzyme replacement therapy, which eventually is expected to lead to an improvement of the digestions and thus of the nutritional status.

The main efforts you are requested concern following the diet, taking a fixed dose of enzymatic supplements and collection of stools.

Your Cystic Fibrosis Unit has already explained to you that you are asked to follow the STUDY PROTOCOL in each stage, which is designed to provide you guidance and support while carrying it out. It is designed to be clear and avoid doubts. It is structured in five days, and it acts at the same time as a handbook and a diary. Sometimes you will just have to carefully look at the test meals preparation and sometimes you will have to answer questions. It is of upmost importance that you follow the instructions strictly, and in the event of an incidence, the document is designed to report it.

We take the opportunity to wish you the highest comfort as possible while doing the test. Finally we encourage you to stick to the protocol during the days of study, and if you feel you need to contact your Cystic Fibrosis Unit for an unforeseen event you do not know how to manage at any of the stages, please contact them through the following telephone number: **000000000**. They will be available from #h to #h.

Yours sincerely,

MyCyFAPP team

BEFORE YOU START

WHAT DO I HAVE TO DO?

DURING THE NEXT THREE DAYS* YOUR MISSION IS TO COMPLY WITH THE FOLLOWING:

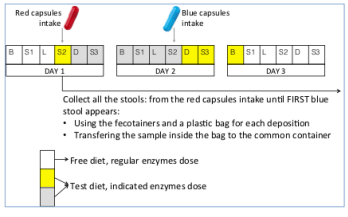
MEALS	Eat what and how much you see in the pictures and indicate afterward how much you ate of the recommended picture + the time you ate this. Do all your best to comply with what is indicated Answer some questions after eating Indicate some medication after eating
ENZYMES	Take the amount of enzymes indicated (PILLBOX) at every mealtimes
COLOUR CAPSULES	Take the colorimetric pills when indicated + indicate the time
STOOLS	Register the information about stools Collect the stools according to the schedule and in separate bags * Keep on collecting until you find the blue colour in your stools

It is of upmost importance that you intake the enzyme capsule(s) **just before** start eating your meal.

CAN I SEE AN OVERVIEW?

- **DAY 1** (e.g. Friday) is the day you start with the study. You can have a free diet and take your regular enzymes doses until the afternoon. The afternoon snack is the first meal of the test. With it, you have to take the indicated amount of enzymes. From this moment on, you have to collect all the stools according to the procedures explained later on.
- **DAY 2** (e.g. Saturday) is the second day of the test. From breakfast to night snack you have to eat what is indicated for all the 6 meals. The enzyme doses for each meal correspond to the compartments of the pillbox. You will see it in the indications. In the afternoon snack, additionally, you will take the blue capsules. You will keep on collecting stools according to the instructions.
- **DAY 3** (e.g. Sunday) is the third and last day of the test. Only at breakfast you will still have to eat what is indicated, and also to take the indicated dose of enzymes. From then on, you can go on with your regular diet and enzymes doses. However, you have to keep on collecting and reporting stools until the blue colour is found.
- **DAY 4 and 5:** the test diet is already finished but the blue stools might have not appeared yet, so you should keep collecting your stools. Thus, we provide you

with the proper space for you to keep on reporting about stools collection during two more days.



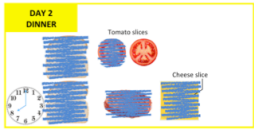
HOW DO I COMPLETE THE DIARY?

You should keep the diary with you during the study days.

Around meal times:

1. Look at the picture to prepare the meal according to indications
2. Take the indicated amount of enzymes (very important !)
3. Paint the portion of the foods you have eaten (ideally, eat them entirely)
4. Answer the questions about the meal (very important !)
5. Register the medication you have taken (if any)

EXAMPLE:



When you go to the toilet:

1. Register the information in the stools registry of each day
EXAMPLE:

HOW DO I COLLECT STOOLS?

You are provided with three devices to collect stools: fecotainers, individual plastic bags and the white container.

- Collect **all** the stools comprised between:

FIRST STOOL COLLECTED	The first stool after you take the red capsules
LAST STOOL COLLECTED	The first stool that is blue dyed

- Follow the procedure shown in the picture below to collect the stools

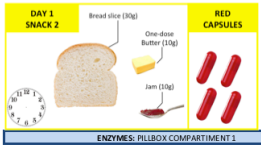
1. Place the fecotainer in the toilet
2. Fit the plastic bag inside it
3. (...)
4. Close the plastic bag when finished
5. Paste the sticker corresponding to the number of deposition
6. Put the plastic bag inside the white container
7. Keep the white container in the freezer
8. Repeat the process until you find the blue stools

- Stickers to identify the stools

- The first stools after the red capsules intake will be labelled with sticker #1
- The following stools will be labelled consecutively along all the study days: #2, #3, etc.
- The first blue stools will be last stool to be collected and labelled.

Example:
Day 1 at 20:00h: stools → #1
Day 2 at 11:00h: stools → #2
Day 2 at 18:00h: stools → #3
Day 3 at 12:00h: stools → #4
Day 3 at 22:00h: BLUE stools → #5 → END of collection

DAY 1

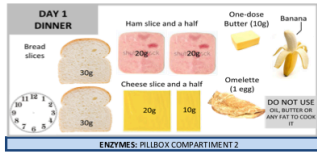


ENZYMES: PILLBOX COMPARTIMENT 1

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

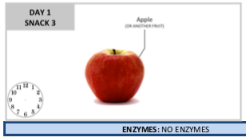


ENZYMES: PILLBOX COMPARTIMENT 2

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	



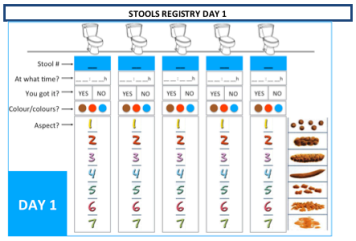
ENZYMES: NO ENZYMES

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

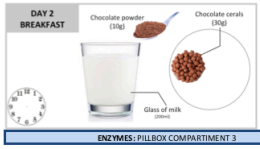
MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

STOOLS REGISTRY DAY 1



OBSERVATIONS

DAY 2

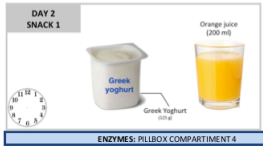


ENZYMES: PILLBOX COMPARTIMENT 3

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

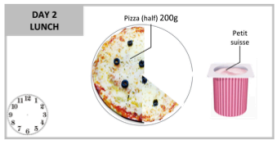


ENZYMES: PILLBOX COMPARTIMENT 4

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

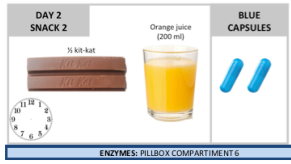


ENZYMES: PILLBOX COMPARTIMENT 5

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

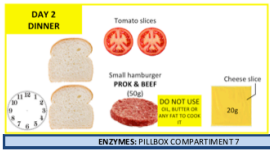


ENZYMES: PILLBOX COMPARTIMENT 6

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

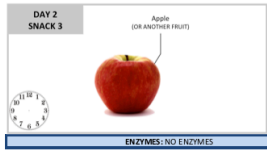


ENZYMES: PILLBOX COMPARTIMENT 7

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	



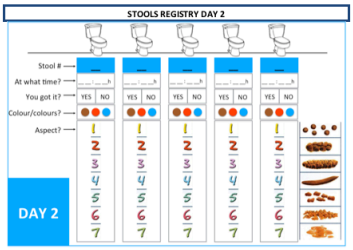
ENZYMES: NO ENZYMES

QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

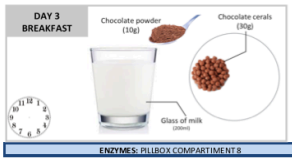
MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

STOOLS REGISTRY DAY 2



OBSERVATIONS

DAY 3



ENZYMES: PILLBOX COMPARTMENT 8

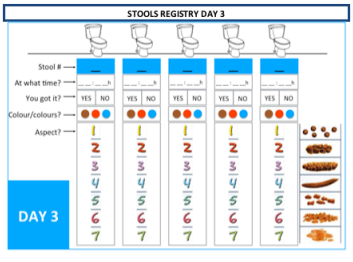
QUESTIONS	YES	NO
Have you taken the indicated amount of enzymes?		
If not, which amount have you taken?		
Have you finished all your plate? If you haven't, paint the amount you have eaten in the picture above		
Were you still hungry?		
Have you eaten anything else? If you have, please indicate it below		
Have you felt abdominal pain or discomfort after the meal?		
How many glasses of water have you taken?		

EXTRA INTAKE? Fruit | Jelly | Ice-lolly | other (?)

MEDICATION					
URSO		Omeprazol		Macrogol	
Domperidone		Antibiotics		Other	

CONGRATULATIONS! You have now finished with the test menu! Please go on with your regular diet and enzymes doses.
Now it's only a matter of collecting the remaining stools until you collect the first blue-dyed stools.

STOOLS REGISTRY DAY 3

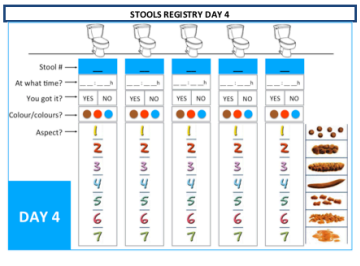


OBSERVATIONS

Have you collected the first blue-dyed stools?
YES: you can stop collecting the stools; you are ready with the protocol!
NO: continue collection and continue on the next pages.

DAY 4

STOOLS REGISTRY DAY 4



OBSERVATIONS

Have you collected the first blue-dyed stools?
YES: you can stop collecting the stools; you are ready with the protocol!
NO: continue collection and continue on the next pages.

ANNEX 7: Test menu used in the pilot study

DAY 1						DAY 2						DAY 3						DAY 4					
B	S1	L	S2	D	S3	B	S1	L	S2	D	S3	B	S1	L	S2	D	S3	B	S1	L	S2	D	S3

free diet
washout
test menu
red capsule intake
blue capsule intake

Level 1

	Item	Amount (x)	Energy	Prot	CH	Lip	SFA	Fibre
Snack 2 day 1	Bread	0,30	63,6	11,2	0,4	1,9	0,7	0,0
	Jam	0,20	29,2	0,1	7,4	0,0	0,0	0,0
	Butter	0,10	74,2	0,1	0,0	8,2	5,1	0,0
	RED CAPS		**167,0**	**11,4**	**7,8**	**10,2**	**5,8**	**0,0**
Dinner day 1	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Ham	0,30	31,8	5,6	0,2	1,0	0,3	0,0
	Omelette	0,60	90,0	7,6	0,2	6,5	1,7	0,0
	Butter	0,10	74,2	0,06	0,04	8,2	10,2	0
	Cheese	0,20	81,4	4,8	0	6,8	4,62	0
	Bananna	0,90	81,0	0,9	17,1	0,2	0,1	1,8
			504,2	**24,6**	**45,0**	**23,9**	**16,9**	**2,8**
Snack 3 day 1	Apple	1,20	64,9	0,4	13,7	0,5	0,0	2,4
			64,9	**0,4**	**13,7**	**0,5**	**0,0**	**2,4**
Breakfast day 2	Full-fat milk (3,6% o	2,00	128,0	6,2	9,2	7,2	4,8	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Chocolate cereals	0,30	114,3	2,3	22,2	1,2	0,5	2,6
			274,7	**9,7**	**35,8**	**8,8**	**5,7**	**5,0**
Snack 1 day 2	Orange juice	1,00	45,0	0,6	10,5	0,0	0,0	0,1
	Greek yoghurt	1,25	153,8	4,6	4,9	12,5	7,8	0,0
			198,75	**5,23**	**15,38**	**12,50**	**7,75**	**0,10**
Lunch day 2								
	Ham & Cheese pizza	1,70	418,2	16,7	44,2	12,8	6,6	0,0
	Petit suisse	0,55	59,95	3,465	7,9013	1,5015	0,8415	0
			478,2	**20,1**	**52,1**	**14,3**	**7,5**	**0,0**
	Kit-kat	0,11	57,0	0,8	7,2	2,9	2,0	0,1
Snack 2 day 2	Fruit juice	1,00	45,0	0,6	10,5	0,0	0,0	0,1
	BLUE CAPS		**102,0**	**1,4**	**17,7**	**2,9**	**2,0**	**0,2**
Dinner day 2	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Hamburger (prok & b	0,50	183,5	11,3	12,4	5,4	0,9	0,0
	Cheese	0,20	81,4	4,8	0,0	6,8	4,6	0,0
	Tomato	0,20	4,4	0,2	0,7	0,0	0,0	0,3
			415,13	**21,87**	**40,47**	**13,51**	**5,53**	**1,30**
Snack 3 day 2 day 2	Apple	1,20	0,0	0,0	0,0	0,0	0,0	0,0
			0,0	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
Breakfast day 3	Full-fat milk (3,6% o	2,00	128,0	6,2	9,2	7,2	4,8	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,30	114,3	2,3	22,2	1,2	0,5	2,6
			274,7	**9,7**	**35,8**	**8,8**	**5,7**	**5,0**

Total energy	Total prot	Total CH	Total Lipids	Total SFA	Total Fibre
1622,7	**61,4**	**179,6**	**62,8**	**39,8**	**10,5**
	% prot	% CH	% Lip	% SFA	
	15,1	**44,3**	**34,8**	**22,1**	

Level 2

	Item	Amount (x)	Energy	Prot	CH	Lip	SFA	Fibre
Snack 2 day 1	Bread	0,30	63,6	11,2	0,4	1,9	0,7	0,0
	Jam	0,20	29,2	0,1	7,4	0,0	0,0	0,0
	Butter	0,10	74,2	0,1	0,0	8,2	5,1	0,0
	RED CAPS		**167,0**	**11,4**	**7,8**	**10,2**	**5,8**	**0,0**
Dinner day 1	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Ham	0,40	42,4	7,5	0,2	1,3	0,4	0,0
	Omelette	0,60	90,0	7,6	0,2	6,5	1,7	0,0
	Butter	0,10	74,2	0,06	0,04	8,2	5,1	0
	Cheese	0,30	122,1	7,2	0	10,2	6,93	0
	Petit Suisse	0,55	60,0	3,5	7,9	1,5	0,8	0,0
			534,5	**31,4**	**35,8**	**29,0**	**15,0**	**1,0**
Snack 3 day 1	Apple	1,20	64,9	0,4	13,7	0,5	0,0	2,4
			64,9	**0,4**	**13,7**	**0,5**	**0,0**	**2,4**
Breakfast day 2	Full-fat milk (3,6% o	2,00	128,0	6,2	9,2	7,2	4,8	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,30	114,3	2,3	22,2	1,2	0,5	2,6
			274,7	**9,7**	**35,8**	**8,8**	**5,7**	**5,0**
Snack 1 day 2	Orange juice	1,00	45,0	0,6	10,5	0,0	0,0	0,1
	Greek yoghurt	1,25	153,8	4,6	4,9	12,5	7,8	0,0
			198,75	**5,23**	**15,38**	**12,50**	**7,75**	**0,10**
Lunch day 2								
	Ham & Cheese pizza	2,20	541,2	21,6	57,2	16,5	8,6	0,0
	Petit Suits	0,55	59,95	3,465	7,9013	1,5015	0,8415	0
			601,2	**25,0**	**65,1**	**18,0**	**9,4**	**0,0**
	Kit-kat	0,22	114,0	1,5	14,3	5,7	4,0	0,2
Snack 2 day 2	Fruit juice	1,00	45,0	0,6	10,5	0,0	0,0	0,1
	BLUE CAPS		**159,0**	**2,1**	**24,8**	**5,7**	**4,0**	**0,3**
Dinner day 2	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Hamburger (prok & b	0,50	183,5	11,3	12,4	5,4	0,9	0,0
	Cheese	0,20	81,4	4,8	0,0	6,8	4,6	0,0
	Tomato	0,20	4,4	0,2	0,7	0,0	0,0	0,3
			415,13	**21,87**	**40,47**	**13,51**	**5,53**	**1,30**
Snack 3 day 2 day 2	Apple	1,20	0,0	0,0	0,0	0,0	0,0	0,0
			0,0	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
Breakfast day 3	Full-fat milk (3,6% o	2,00	128,0	6,2	9,2	7,2	4,8	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,30	114,3	2,3	22,2	1,2	0,5	2,6
			274,7	**9,7**	**35,8**	**8,8**	**5,7**	**5,0**

Total energy	Total prot	Total CH	Total Lipids	Total SFA	Total Fibre
1832,9	**73,9**	**190,6**	**74,4**	**41,8**	**8,9**
	% prot	% CH	% Lip	% SFA	
	16,1	**41,6**	**36,6**	**20,5**	

Level 3

	Item	Amount (x)	Energy	Prot	CH	Lip	SFA	Fibre
Snack 2 day 1	Bread	0,30	63,6	11,2	0,4	1,9	0,7	0,0
	Jam	0,20	29,2	0,1	7,4	0,0	0,0	0,0
	Butter	0,10	74,2	0,1	0,0	8,2	5,1	0,0
	RED CAPS		**167,0**	**11,4**	**7,8**	**10,2**	**5,8**	**0,0**
Dinner day 1	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Ham	0,40	42,4	7,5	0,2	1,3	0,4	0,0
	Omelette	1,20	180,0	15,1	0,5	13,0	3,4	0,0
	Butter	0,10	74,2	0,06	0,04	8,2	5,1	0
	Cheese	0,40	162,8	9,6	0	13,6	9,24	0
	Bananna	0,90	81,0	0,9	17,1	0,2	0,1	1,8
			686,2	**38,8**	**45,3**	**37,5**	**18,2**	**2,8**
Snack 3 day 1	Apple	1,20	64,9	0,4	13,7	0,5	0,0	2,4
			64,9	**0,4**	**13,7**	**0,5**	**0,0**	**2,4**
Breakfast day 2	Full-fat milk (3,6% o	2,50	160,0	7,8	11,5	9,0	6,0	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,50	190,5	3,9	37,0	2,0	0,9	4,4
			382,9	**12,9**	**52,9**	**11,4**	**7,3**	**6,8**
Snack 1 day 2	Orange juice	2,00	90,0	1,2	21,0	0,0	0,0	0,2
	Greek yoghurt	1,25	153,8	4,6	4,9	12,5	7,8	0,0
			243,75	**5,83**	**25,88**	**12,50**	**7,75**	**0,20**
Lunch day 2								
	Ham & Cheese pizza	2,20	541,2	21,6	57,2	16,5	8,6	0,0
	Petit Suisse	0,55	59,95	3,465	7,9013	1,5015	0,8415	0
			601,2	**25,0**	**65,1**	**18,0**	**9,4**	**0,0**
	Kit-kat	0,33	170,9	2,3	21,5	8,6	5,9	0,3
Snack 2 day 2	Fruit juice	1,00	45,0	0,6	10,5	0,0	0,0	0,1
	BLUE CAPS		**215,9**	**2,9**	**32,0**	**8,6**	**5,9**	**0,4**
Dinner day 2	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Hamburger (prok & b	0,90	330,3	20,3	22,2	9,6	1,6	0,0
	Cheese	0,20	81,4	4,8	0,0	6,8	4,6	0,0
	Tomato	0,20	4,4	0,2	0,7	0,0	0,0	0,3
			561,93	**30,87**	**50,35**	**17,79**	**6,25**	**1,30**
Snack 3 day 2 day 2	Apple	1,20	0,0	0,0	0,0	0,0	0,0	0,0
			0,0	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
Breakfast day 3	Full-fat milk (3,6% o	2,50	128,0	6,2	9,2	9,0	4,8	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,50	114,3	2,3	22,2	2,0	0,5	2,6
			274,7	**9,7**	**35,8**	**11,4**	**5,7**	**5,0**

Total energy	Total prot	Total CH	Total Lipids	Total SFA	Total Fibre
2194,9	**85,8**	**234,8**	**88,5**	**48,6**	**12,6**
	% prot	% CH	% Lip	% SFA	
	15,6	**42,8**	**36,3**	**19,9**	

Level 4

	Item	Amount (x)	Energy	Prot	CH	Lip	SFA	Fibre
Snack 2 day 1	Bread	0,30	63,6	11,2	0,4	1,9	0,7	0,0
	Jam	0,20	29,2	0,1	7,4	0,0	0,0	0,0
	Butter	0,10	74,2	0,1	0,0	8,2	5,1	0,0
	RED CAPS		**167,0**	**11,4**	**7,8**	**10,2**	**5,8**	**0,0**
Dinner day 1	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Ham	0,40	42,4	7,5	0,2	1,3	0,4	0,0
	Omelette	1,20	180,0	15,1	0,5	13,0	3,4	0,0
	Butter	0,10	74,2	0,06	0,04	8,2	5,1	0
	Cheese	0,40	162,8	9,6	0	13,6	9,24	0
	Bananna	0,90	81,0	0,9	17,1	0,2	0,1	1,8
			686,2	**38,8**	**45,3**	**37,5**	**18,2**	**2,8**
Snack 3 day 1	Apple	1,20	64,9	0,4	13,7	0,5	0,0	2,4
	Greek yoghurt	1,25	153,8	4,6	4,9	12,5	7,8	0,0
			218,7	**5,0**	**18,6**	**13,0**	**7,8**	**2,4**
Breakfast day 2	Full-fat milk (3,6% o	2,50	160,0	7,8	11,5	9,0	6,0	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,50	190,5	3,9	37,0	2,0	0,9	4,4
			382,9	**12,9**	**52,9**	**11,4**	**7,3**	**6,8**
Snack 1 day 2	Orange juice	2,00	90,0	1,2	21,0	0,0	0,0	0,2
	Greek yoghurt	1,25	153,8	4,6	4,9	12,5	7,8	0,0
			243,75	**5,83**	**25,88**	**12,50**	**7,75**	**0,20**
Lunch day 2								
	Ham & Cheese pizza	2,50	615,0	24,5	65,0	18,8	9,8	0,0
	Petit Suisse	1,00	109	6,3	14,366	2,73	1,53	0
			724,0	**30,8**	**79,4**	**21,5**	**11,3**	**0,0**
	Kit-kat	0,44	227,9	3,1	28,6	11,4	7,9	0,4
Snack 2 day 2	Fruit juice	2,00	90,0	1,2	21,0	0,0	0,0	0,2
	BLUE CAPS		**317,9**	**4,3**	**49,6**	**11,4**	**7,9**	**0,6**
Dinner day 2	Bread	0,60	145,8	5,6	27,4	1,3	0,0	1,0
	Hamburger (prok & b	0,90	330,3	20,3	22,2	9,6	1,6	0,0
	Cheese	0,20	81,4	4,8	0,0	6,8	4,6	0,0
	Tomato	0,20	4,4	0,2	0,7	0,0	0,0	0,3
			561,93	**30,87**	**50,35**	**17,79**	**6,25**	**1,30**
Snack 3 day 2 day 2	Apple	1,20	0,0	0,0	0,0	0,0	0,0	0,0
			0,0	**0,0**	**0,0**	**0,0**	**0,0**	**0,0**
Breakfast day 3	Full-fat milk (3,6% o	2,50	128,0	6,2	9,2	9,0	4,8	0,0
	Cocoa powder	0,10	32,4	1,2	4,4	0,4	0,4	2,4
	Nesquick cereals	0,30	114,3	2,3	22,2	1,2	0,5	2,6
			274,7	**9,7**	**35,8**	**10,6**	**5,7**	**5,0**

Total energy	Total prot	Total CH	Total Lipids	Total SFA	Total Fibre
2573,4	**97,5**	**271,6**	**107,3**	**60,2**	**12,8**
	% prot	% CH	% Lip	% SFA	
	15,2	**42,2**	**37,5**	**21,0**	


UNIVERSITAT
POLITÈCNICA
DE VALÈNCIA


IIAD


Instituto de Investigación
Sanitaria La Fe


MyCyFAPP