UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE BIOTECNOLOGÍA

STUDY OF THE EFFECT OF HYPOXIA ON ACTIVATION, DEGRANULATION AND OXIDATIVE PROFILE OF NEUTROPHILS IN PATIENTS WITH ALPHA-1 ANTITRYPSIN DEFICIENCY

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Abstract and keywords

Abstract:

The number of neutrophils in the lungs of patients with alpha 1 antitrypsin deficiency (AATD) is significantly higher than in those of healthy individuals, which could contribute to an increase in proteolytic activity and a greater damage in lung tissue, thus promoting the development of emphysema observed in some of the patients. We considered the overall objective of this research project to determine if hypoxia induces the activation of neutrophils in patients with AATD. According to our working hypothesis, tissue hypoxia would produce the activation of neutrophils, with the consequent release to the tissue environment of its proteinase content, which would increase its ability to damage lung tissue. On the other hand, the prolongation of neutrophil half-life caused by hypoxia will increase lung tissue exposure to activated neutrophils, which will contribute to the development of the inflammatory process through the release of inflammatory cytokines. The increase in the number of neutrophils in the lungs will contribute to decrease the local O2 levels, leading to an increase in the reactive oxygen species (ROS) quantity and a situation of oxidative damage in the biomolecules (DNA, lipids and proteins) that will contribute to the development of the damage in the lung tissue. In patients with AATD, the neutrophil-released proteinases and the increase in ROS is not eliminated correctly due to the existence of low plasma levels of alpha 1 antitrypsin (AAT). Based on the working hypothesis, we propose as specific objectives: a) Develop a protocol for a long-term study of the degranulation, oxidative profile, ROS production and gene expression in neutrophils from AATD patients and healthy volunteers. b) The determination of the differences of the previous parameters in neutrophils from healthy volunteers against different phenotypes of patients with AATD. The results show that hypoxia positively affects neutrophils degranulation and produces changes in the oxidative status and gene expression of hypoxia-inducible factors. Despite the targets of the study were pediatric patients from a rare disease and limitations in the number of patients impede the collection of robust data, the goal of creating a protocol for studying degranulation, oxidative damage and gene expression variations was successfully achieved.

Keywords:

Reactive oxygen species, neutrophil, alpha 1 antitrypsin deficiency, hypoxia.

Datos del proyecto

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Resumen y palabras clave

Resumen:

El número de neutrófilos en los pulmones de pacientes con déficit de alfa 1 antitripsina (DAAT) es significativamente más alto que en individuos sanos, lo que podría contribuir a un aumento de la actividad proteolítica y un mayor daño en el tejido pulmonar, promoviendo así al desarrollo de enfisema observado en algunos de los pacientes. Consideramos que el objetivo general de este proyecto de investigación es determinar si la hipoxia induce la activación de neutrófilos en pacientes con DAAT. De acuerdo con nuestra hipótesis de trabajo, la hipoxia tisular produciría la activación de neutrófilos, con la consecuente liberación al contenido tisular de proteinasas, lo que aumentaría su capacidad de dañar el tejido pulmonar. Por otro lado, la prolongación de la vida media de los neutrófilos causada por la hipoxia aumentaría la exposición del tejido pulmonar a los neutrófilos activados, lo que puede contribuir al desarrollo del proceso inflamatorio a través de la liberación de citoquinas inflamatorias. El aumento en el número de neutrófilos pulmonares contribuirá a disminuir los niveles locales de O_2, lo que lleva a un aumento en la cantidad de especies reactivas de oxígeno (ERO) y una situación de daño oxidativo en las biomoléculas (ADN, lípidos y proteínas) que contribuirán al desarrollo del daño en el tejido pulmonar. En pacientes con DAAT, las proteinasas liberadas por neutrófilos y el aumento de ERO no se eliminan correctamente debido a la existencia de niveles plasmáticos bajos de alfa 1 antitripsina. Con base en la hipótesis de trabajo, proponemos como objetivos específicos: a) Desarrollar un protocolo para un estudio a largo plazo de la degranulación, el perfil oxidativo, la producción de ROS y la expresión génica en neutrófilos de pacientes con AATD y voluntarios sanos. b) La determinación de las diferencias de los parámetros previos en neutrófilos de voluntarios sanos frente a diferentes fenotipos de pacientes con AATD. Los resultados muestran que la hipoxia afecta positivamente a la degranulación de neutrófilos y produce cambios en el estado oxidativo y la expresión génica de factores inducibles por hipoxia. A pesar de que los objetivos del estudio fueron pacientes pediátricos de una enfermedad rara y las limitaciones en el número de pacientes impiden la recopilación de datos sólidos, se logró con éxito el objetivo de crear un protocolo para estudiar la degranulación, el daño oxidativo y las variaciones de expresión génica.

Palabras clave:

Especies reactivas de oxígeno, neutrófilos, déficit de alfa 1 antitripsina, hipoxia**.**

Dates del projecte

Títol: Estudi de l'efecte de la hipòxia en l'activació, degranulació i perfil oxidatiu en neutròfils de pacients amb dèficit d'alfa 1 antitripsina

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Resum i paraules clau

Resum:

El nombre de neutròfils en els pulmons de pacients amb dèficit d'alfa 1 antitripsina (DAAT) és significativament més alt que en individus sans, el que podria contribuir a un augment de l'activitat proteolítica i un major dany en el teixit pulmonar, promovent així el desenvolupament d'emfisema observat en alguns dels pacients. Considerem que l'objectiu general d'aquest projecte d'investigació és determinar si la hipòxia indueix l'activació de neutròfils en pacients amb DAAT. D'acord amb la nostra hipòtesi de treball, la hipòxia tissular produiria l'activació de neutròfils, amb la conseqüent alliberament al contingut tissular de proteïnases, el que augmentaria la seva capacitat de danyar el teixit pulmonar. D'altra banda, la prolongació de la vida mitjana dels neutròfils causada per la hipòxia augmentaria l'exposició del teixit pulmonar als neutròfils activats, el que pot contribuir al desenvolupament del procés inflamatori a través de l'alliberament de citocines inflamatòries. L'augment en el nombre de neutròfils pulmonars contribuirà a disminuir els nivells locals d'O_2, el que porta a un augment en la quantitat d'espècies reactives d'oxigen (ERO) i una situació de dany oxidatiu en les biomolècules (ADN, lípids i proteïnes) que contribuiran al desenvolupament del dany en el teixit pulmonar. En pacients amb DAAT, les proteïnases alliberades per neutròfils i l'augment d'ERO no s'eliminen correctament causa de l'existència de nivells plasmàtics baixos d'alfa 1 antitripsina. Amb base a la hipòtesi de treball, proposem com a objectius específics: a) Desenvolupar un protocol per a un estudi a llarg termini de la degranulació, el perfil oxidatiu, la producció de ROS i l'expressió gènica en neutròfils dels pacients amb AATD i voluntaris sans. b) La determinació de les diferències dels paràmetres previs en neutròfils procedents de voluntaris sans davant diferents fenotips de pacients amb AATD. Els resultats mostren que la hipòxia afecta positivament la degranulació dels neutròfils i produeix canvis en l'estat oxidatiu i l'expressió gènica dels factors induïbles per la hipòxia. Tot i que els objectius de l'estudi eren pacients pediàtrics d'una malaltia poc freqüent i les limitacions en el nombre de pacients impedien la recollida de dades robustes, es va aconseguir assolir l'objectiu de crear un protocol per a l'estudi de la degranulació, el dany oxidatiu i les variacions d'expressió gènica.

Paraules clau:

Espècies reactives d'oxigen, neutròfils, dèficit d'alfa 1 antitripsina, hipòxia.

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Declaration

This dissertation has been composed by the candidate, Daniel Pellicer Roig, and I confirm that the work presented is my own, except where otherwise stated.

The candidate presented this project in the "Jornada de jóvenes investigadores en enfermedades raras"; the conference took place in Valencia, Spain on date February 27, 2018.

Daniel Pellicer Roig

Index

Figure's index

Table's index

Equations index

Abbreviations

8-OHdG	8-hydroxy-2'–deoxyguanosine
A	Adenosine
AAT	Alpha-1 antitrypsin
AATD	Alpha-1 antitrypsin deficiency
ATS	American thorax society
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
BSA	Bovine serum albumin
cDNA	Complementary DNA
CMFDA	5-chloromethylfluorescein diacetate
COPD	Chronic obstructive pulmonary disease
Ct	Cycle threshold
DAF-FM	4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DCFH	2' 7-Dichlorohydrofluorescein
DEM	Diethylmaleate
DHR	1,2,3 - Dihydrorhodamine
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERS	European respiratory society
Fc	Fragment crystallizable
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
fMLP	Formylated peptide
FSC	Forward scatter
FTC	Fluorescein-5-thiosemicarbazide
FU	Fluorescent units
G	Guanine
GM-CSF	Granulocyte and macrophages colony stimulated factors
GSH	Reduced glutathione
h	Hour(s)
H_2O_2	Oxygen peroxide
HCC	Hepatocarcinoma
HClO	Hypochlorous acid
HE	Hydroethidine
IL	Interleukin
ILRA	Interleukin receptor agonist
IUBMB	International Union of Biochemistry and Molecular Biology
MCB	Monochlorobimane
MDA	Malondialdehyde
min	Minute(s)
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate

NET	Neutrophilic extracellular trap
NF-kβ	Nuclear factor kappa B
NO	Nitric oxide
NOR-1	(+/-)-(E)-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide
O/N	Overnight
O_2^-	Anion radical superoxide
OD	Optical density
OMIM	Online Mendelian inheritance in man
$ONOO^-$	Peroxynitrites
PAF	Platelet activating factor
PAS	Periodic acid Schiff
PB	5-hydroxy-2-methyl-1,4-naphthoquinone
PBS	Phosphate buffered saline
PI	Propidium iodine
PS	Phosphatidylserine
qPCR	Quantitative polymerase chain reaction
REDAAT	Spanish registry of patients with alpha 1 antitrypsin deficit
RER	Rough endoplasmic reticulum
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription - quantitative polymerase chain reaction
s	Second(s)
SD	Standard deviation
SEM	Standard error of the mean
SEPAR	Spanish society of pneumology and thoracic surgery
SERPINA	Serine protease inhibitor
SOD	Superoxide dismutase
SSC	Side scatter
T	Thymine
TIMP	Tissue inhibitor of metalloproteinase
TMB	3,3',5,5'-Tetramethylbenzidine
TMRM	Tetramethylrhodamine methyl ester perchlorate
TNF-α	Tumor necrosis factor alpha
α1-Pi	Alpha-1 proteinase inhibitor
ΔΔCt	Delta-delta cycle threshold
$Δψ_m$	Membrane potential
λem	Maximum wavelength of emission
λex	Maximum wavelength of excitation

1. Introduction

1.1. Alpha-1 antitrypsin

Alpha-1 antitrypsin (AAT), also known as alpha-1 proteinase inhibitor (α1-Pi) or SERPINA1 (Serine protease inhibitor group A, member 1) is a mean size (6.7 x 3.2 nm) circulatory glycoprotein. Its molecular weight is 52 kDa and has a mean average half-life of 5 days. It has the capability to spread along the tissues due to its hydrosoluble properties (Hill and Kalsheker, 2017).

1.2. Structure

AAT is initially transcribed as a 418 amino acids immature protein. Once secreted, it undergoes a maturation process in the Golgi apparatus, where its 24 amino acids signaling peptide are removed. After that modification, three carbohydrate strains join the molecule anchoring the asparagine residues present. This final conformation acquires a tridimensional structure formed by 9 α-helixes (named with capital letters A-I) and 15 β-lamina named:

- 5 vertical bands between the superior and inferior pole (A1-5).
- 6 horizontal lamina placed in the middle (B1-6).
- 4 tilted bands in the upper structure zone (C1-4).
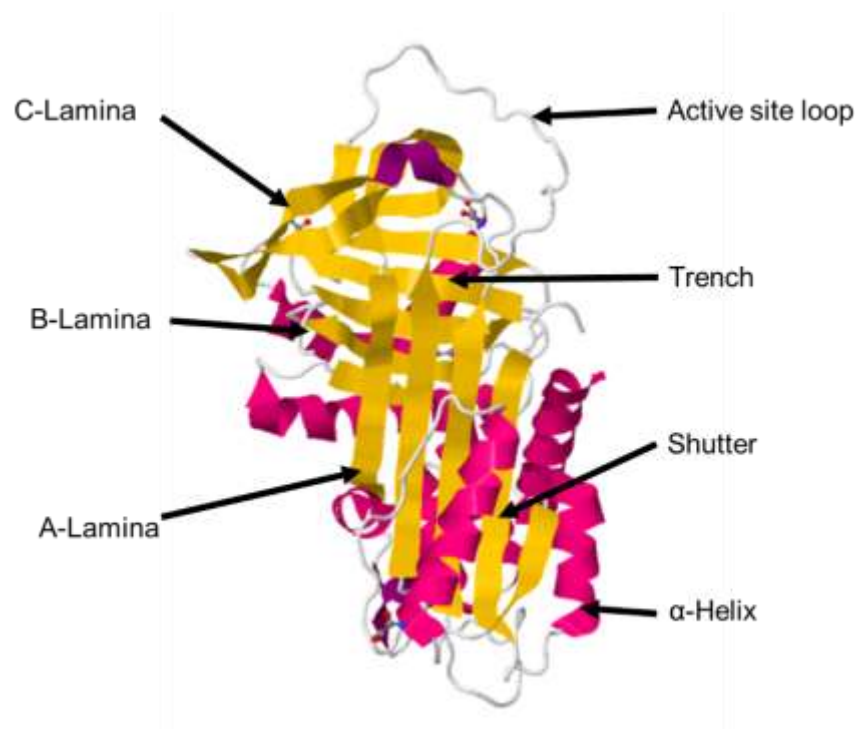
Figure 1. 3D representation of the alpha 1 antitrypsin molecule. All the significant parts are labeled and indicated. PDB ID 5NBU, Resolution: 1.67 Å.

This final structure creates a compact shield, which holds in its interior a 20 residues mobile loop that contains the active site. The active site function is held by a methionine and a serine in the 358 and 359 position respectively. It faces the exterior part of the protein, which allows

the creation of the bound with the substrate. In the core of the protein, two different indentations can be found: the "breach" and the "shutter". Those regions hold most of the physiological and mutational changes that modify the properties of the AAT (Lomas and Parfrey, 2004) (Figure 1).

1.3. Gene

The 12.2 kilobases gene is situated in the distal extreme of the chromosome 14 (position q31-32.3). It shares locus with other serpina-codifying genes and a pseudogene (Janciauskiene et al., 2011). In the Online Mendelian Inheritance in Man (OMIM) classification it has the code 613490 (locus MIM number 107400 (Source: www.omim.org). This protein is codified by 7 different exons (named with the roman numbers I-VII, (IA,IB, IC, II, III, IV, V)) separated by 6 interspace sequences. Each exon is important for the codification of different parts of the protein and exons have been found to be dependent on the cell-type. Exons IA and B codify the initial signal in the macrophage transcription. Meanwhile, IC does it in the hepatocyte (Lai et al., 1983).

Polymorphisms are characteristic of the AAT gene. Genotyping techniques have permitted the identification of more than 125 variants that differ in the quantity of protein available in the serum. Among them, it is possible to find variants with normal, deficit and null levels of AAT (Lee and Brantly, 2000; Salahuddin, 2010; de Serres and Blanco, 2014).

1.4. Synthesis and secretion.

AAT is synthesized mainly by the hepatocytes (approximately 80% of the total), but additional monocytes, neutrophils, macrophages, alpha and delta pancreatic cells, alveoli pulmonary cells and endothelial cells can produce additional quantities. Protein production is on average 34 mg·day^{-1}·kg^{-1} of corporal weight, which supposes around 1 or 2 grams of protein per liter of plasma. AAT can reach higher concentrations when different stimuli affect the homeostasis of the organism, and the serum levels concentration can be multiplied by 2 or even up to 5 during inflammatory or infectious processes. Up to 80% of the protein moves to the interstitial space and between 0.5 to 10% reaches the biological fluids: saliva, tears, milk (0.3-0.6 g/L), semen, urine, alveolar liquid (0.1-0.3 g/L) cephaloraquid liquid (0.006-0.02 g/L) bile (0.005 g/L). (Lomas and Parfrey, 2004).

1.5. Functions and properties of AAT

AAT is the most abundant protease inhibitor and its main function is the inhibition of the elastase produced by neutrophils, its reaction is carried on with one of the highest association constant known in physiology (k = 6.5 x 10^7 M^{-1}s^{-1}) (Vidal et al., 2006). Among other properties, inhibition of serin proteases must be highlighted (Duranton et al., 2000; Spencer et al., 2004; Travis and Salvesen, 1983), as AAT gives to the serum as high as the 90% of its total antiprotease capacity. Other features related to AAT are the inhibition of the intercellular and membrane proteinases (Travis and Salvesen, 1983), non-inhibitory anti-inflammatory properties, immunomodulation (Spencer et al., 2004) and antimicrobial activity (He et al., 2004).

1.6. Inheritance

AATD is inherited in an autosomal codominant manner, which implies that each allele contributes in a 50% to the protein production and, consequently, to the final concentration in blood and tissue (Cohen, 1975). The normal alleles, present in 85-90% of the individuals, receive the name M, for its electrophoretic mobility, so an individual with two normal alleles is named as a MM phenotype.

Among the deficient alleles, the most frequent ones are named by the letters S and Z, present in a 10% and 2% of the Spanish population respectively. The alleles M, S, and Z express around 100, 60 and 15% of AAT respectively. Following this assumption, MM would express 100% of AAT in blood, meanwhile MS, SS, MZ, SZ, and ZZ, express 80, 60, 55, 40, and 15% respectively (Vidal et al., 2006).

1.7. Alpha-1 Antitrypsin Deficiency in the rare diseases field

Alpha-1 Antitrypsin Deficiency (AATD) was described for the first time in 1963 by Laurell and Eriksson, who observed the absence of the α1 band in the protein electrophoresis taken from the patients serum from the Respiratory Department in the MblmG Ccnwal Hospital, Malmö, Lund University, Sweden (Laurell and Eriksson, 1963) (Figure 2).

The deficiency is defined by serum levels under 35% of the medium value. It is an infrequent condition, generally related with the phenotypes ZZ (96% of the pathologies associated with the AATD), and with much less frequency, with the diverse combination of the alleles Z, S, rare or extremely rare (2003).
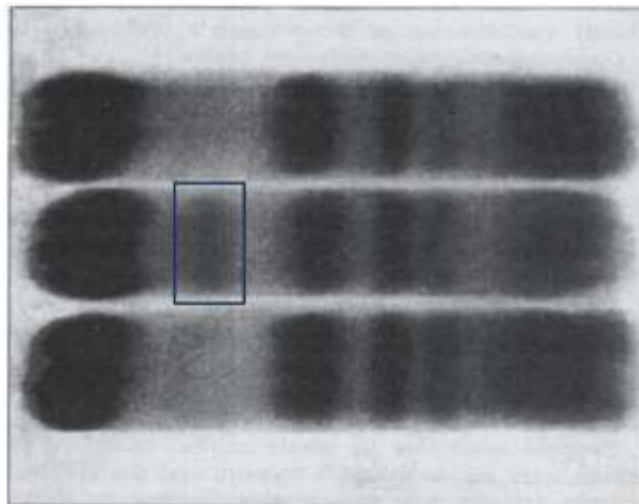
Figure 2. Electrophoretic pattern of the serum of two patients with Alpha-1 Antitrypsin Deficiency (AATD) and a control. The upper and the bottom are the patient's bands. The Alpha-1 Antitrypsin (AAT) band is notably fainted in the patients, while it can be seen in the labeled square in the control (Laurell and Eriksson, 1963)*.*

1.8. Molecular bases of AATD

Inhibitory advantages are conferred to AAT by its complex structure, but this feature is double-edged, as fragility is highly increased. As it is mentioned before, AATD is prone to suffer from conformational changes, allowing the creation of different alleles as S and Z(Hill and Kalsheker, 2017).

S mutation is produced by a nucleotide change in exon III, in which the normal triplet GAA (Guanine-Adenosine-Adenosine) turns into GTA (Guanine-Thymine-Adenosine). After suffering the mutation, translation of the triplet changes from glutamic acid to valine in the position 264 (GAA Glu264 » GTA Val264) of the chain, situated in the "shutter" part of the protein described before (see section 1.2). The location of the "shutter" in the protein, separated from the active site, means that less severe changes appear in the protein structure and it maintains part of its catalytic activity. In addition, there is no evidence of polymerization, only dimerization (Figure 3), which impedes its accumulation in the rough endoplasmic reticulum (RER) (Lomas et al., 1992).
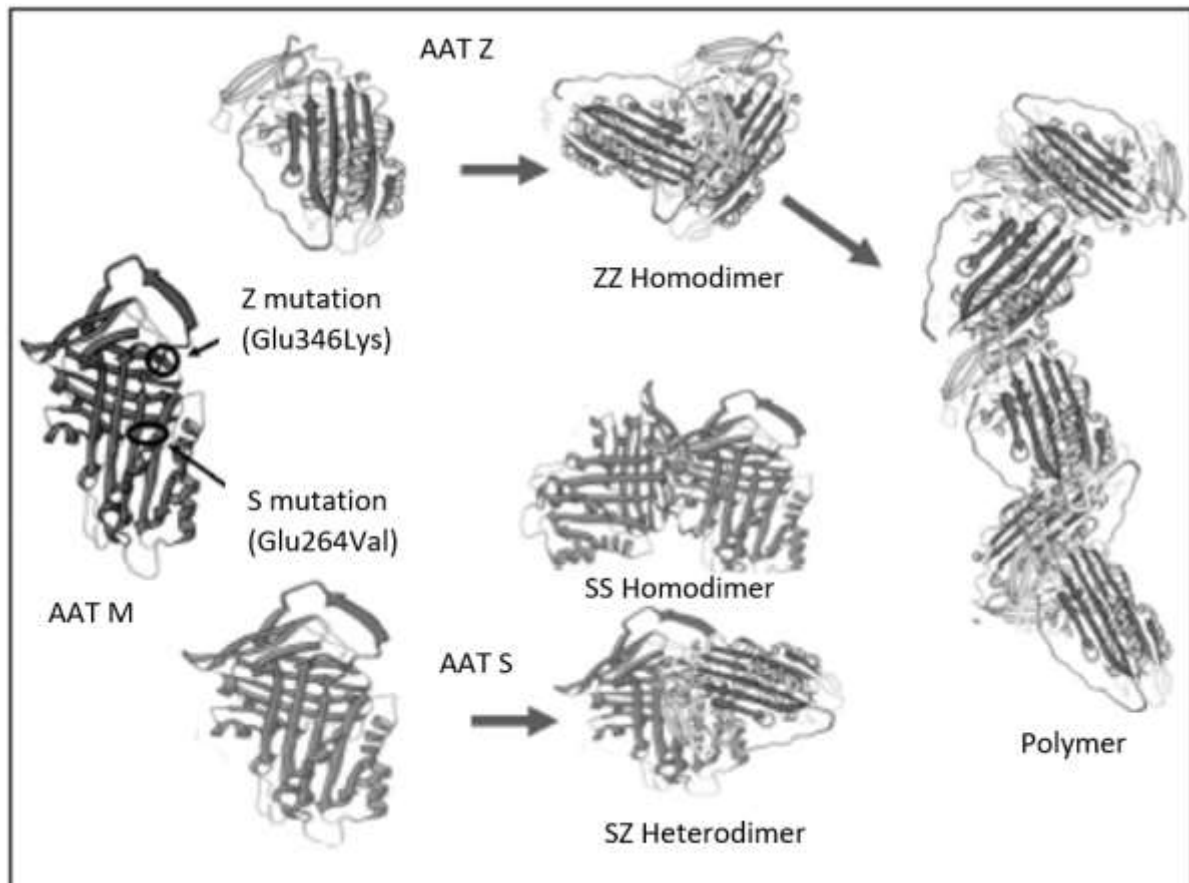
Figure 3. S & Z mutations. Conformational changes and polymerization. Those conformational changes destabilize the proteins and they tend to aggregate with other mutated proteins. This aggregation is produced between the active site and the lamina of the molecule. (Menga et al., 2014) ***AAT** = Alpha 1 antitrypsin.*

Z mutation causes more severe symptoms. In this case, the nucleotidic change occurs in exon V, normally expressed as AAG (Adenine-Adenine-Guanine), and it mutates to GAG (Guanine-Adenine-Guanine). As a consequence, the glutamic acid in the position 342 changes to a lysine (GAG Glu342 » AAG Lys342) which has dramatic changes in the "breach" part of the protein. This amino acid change occurs in the insertion point of the apex of the active site's loop in the upper termination of an A lamina. The hydrogen bonds broken due to this change cause acute widening of both the "breach" and the "shutter", decreasing the protein's stability and leading to pathological effects (Lomas and Mahadeva, 2002; Lomas and Parfrey, 2004; Mahadeva et al., 2005). The more severe effects are observed when protein stability is increased by dimerization or, in ZZ homozygous patients, polymerization, creating insoluble aggregates that damage the inner areas in the cell. The cavity so-called s4A triggers ZZ protein polymerization (Berthelier et al., 2015) (Figure 3).

Another allele of interest in this study is the Mattawa null allele. In this mutation, AAT suffers such a conformational change that allows its detection and degradation by the intracellular machinery before its polymerization. These patients are prone to undergo pulmonary emphysema, but not to develop hepatopathies (Lara et al., 2013).

A considerable amount of alleles have been identified during the past years, and they have been named after the electrophoretic mobility of the protein and/or the place they were discovered. As the total number of mutations have grown in number, genetic studies allowed their description with a standardized nomenclature for the nucleotide and the protein changes. Data of the most common of the rare alleles are presented in the following table (Table 1).

Table 1. AAT mutations. It includes nucleotide nomenclature, and both standardized and legacy protein nomenclature. The original name given to the mutation is presented in the synonyms column.

Nucleotide nomenclature[a]	Protein nomenclature (Unprocessed)	Protein nomenclature (legacy)	Synonyms
17 kb deletion of all coding exons			$Nullisola_{di\ procida}$; $Null_{procida}$
c.17C>T	p.Ser6Leu	p.Ser-19Leu	$Z_{wrexham}$
c.194T>C	p.Leu65Pro	p.Leu41Pro	$M_{procida}$
c.227_229delTCT	p.Phe76del	p.Phe52del	M_{malton}; $M_{palermo}$
c.230C>T	p.Ser77Phe	p.Ser53Phe	S_{iiyama}
c.272G>A	p.Gly91Glu	p.Gly67Glu	$M_{mineral\ springs}$
c.275C>T	p.Thr92ile	p.Thr68ile	$Null_{lisbon}$
c.347T>A	p.Ile116Asn	p.Ile92Asn	$Null_{ludwigshafen}$
c.415G>A	p.Gly139Ser	p.Gly115Ser	$Null_{devon}$; $Null_{Newport}$
c.552delC	p.Tyr184X	p.Tyr160Ter	$Null_{granite\ falls}$
c.646+1G>T			$Null_{west}$
c.721A>T	p.Lys241X	p.Lys217Ter	$Null_{bellingham}$
c.739C>T	p.Arg247Cys	p.Arg223Cys	F
c.839A>T	p.Asp280val	p.Asp256val	P_{duarte}; P_{lowell}; $Null_{cardiff}$
c.863A>T[b]	p.Glu288val	p.Glu264val	S
c.1027_1028delTC	p.Ser343ArgfsX16	p.Ser319ArgfsX16	$Null_{hong\ kong\ 1}$
c.1078G>A	p.Ala360Thr	p.Ala336Thr	$w_{bethesda}$
c.1096G>A[b]	p.Glu366Lys	p.Glu342Lys	Z
1130dupT	p.Leu377PhefsX24	p.Leu353PhefsX24	$Null_{mattawa}$
c.1158delC	p.Glu387ArgfsX11	p.Glu363ArgfsX11	$Null_{bolton}$
c.1158dupC	p.Glu387ArgfsX14	p.Glu363ArgfsX14	$Null_{saarbruecken}$
c.1178C>T	p.Pro393Leu	p.Pro369Leu	$M_{heerlen}$

This table was adapted from: (Abboud et al., 2011)

[a] Nucleotide changes are described relative to NM_001127705.1. [b] The most common SERPINA1 mutations: i) c.863 A>T (or "S") is most common in individuals of Southern European origin, the allele frequency in white subjects in North America is about 6%; ii) c.1096G>A (or "Z") is most common in individuals of Northern European origin, the allele frequency is about 2% in white subjects in North America.

Protein standardized nomenclature use is rising in detriment of protein legacy nomenclature, but both nomenclatures are still used. Gene counseling should be provided to affected families who have been detected with a deficient allele.

1.9. Pathophysiology

AATD is included in the wider group of diseases called serpinopathies, whose pathophysiology depends on the accumulation of an abnormal protein in the cell and low levels of a circulating protein. This accumulation causes cell damage that leads to a posterior cell death, and the clinical manifestations depend on the cell loss in the tissues (Lara, 2010). In the particular case of AATD, the mutated proteins have difficulties to be correctly folded by the AAT producer cells and they remain in their RER, where polymerization starts (Ogushi et al., 1987). The formation of stable inclusion bodies triggers the activation of various protein-cyclases and the cytosolic nuclear factor kappa B (NF-kβ), activating the caspase cascade and stimulating cellular apoptosis (Vidal et al., 2006). Moreover, it has been demonstrated that cell stress induced by inclusion bodies causes mitochondrial damage and activates the autophagy, which can generate fibrosis or hepatic cirrhosis (Sveger, 2017).

Pulmonary emphysema is another pathology related to the ZZ homozygous patients. Nevertheless, its multifactorial mechanism is poorly understood due to plethora factors that seem to participate in it. It is generally accepted that the emphysema is a consequence of the role that neutrophils play in the disease. In ZZ homozygous patients, AAT is forming the inclusion bodies in the RER of the secretory cells, which impedes its secretion to the bloodstream and lowers its concentration in the organism. This low concentration distorts the protease/antiprotease equilibrium and creates a chronic pulmonary process of inflammation-destruction-reparation that threatens organism homeostasis (2003).

1.10. Clinical manifestations in Adult and Pediatric age

AATD clinical manifestations are uncertain and depend of the allele carried by the patient and the exposition to hazardous environmental factors. The symptoms mainly appear at two different stages of life. During pediatric age, it is more feasible to undergo a hepatopathy which might be solved spontaneously (Escribano et al., 2016, Torres-Durán et al., 2018). However, children would not likely undergo pulmonary condition. That fact makes impossible to differentiate between MM and ZZ children because of their behavior. If the condition has not presented hepatic symptoms or a transaminase alteration, it might not be detected. However, with genetic counselling from parents that have been previously diagnosed, it is possible to be aware if their offspring have any possibility of carrying a deficit allele and perform a genetic test if necessary.

In the adult stage, AATD is occasionally discovered through blood analysis when successive respiratory symptoms appear without an apparent link. This finding might not explain or cause the symptomatology, but it could lead to a progression of an underlying respiratory condition that could accelerate the typical AATD damage found in the lungs (Blanco, 2017). The most information about this disease has been reported by long-term studies of pediatric patients found by neonatal genetic sorting (Piitulainen and Sveger, 1998; Sveger, 1984, 2017). One of them, which took part in Sweden in children with the severe phenotype ZZ or intermediate SZ, reported that patient's behavior was similar to the general population. At the age of 8, the frequency of asthma or whistling bronchitis was 8% (not different from the controls). At the age of 16, in the SZ patients, pneumonia without another manifestation was slightly increased. Nevertheless, some of the patients, especially the asthmatic ones, showed an impaired lung function (Sveger, 2017).

What seems clear is that AATD is unlikely to increase the possibility of having pulmonary manifestations on its own in pediatric age, but it makes the pulmonary damage to evolve faster when an external factor is present. Tobacco consumption, pollution, asthma or repeated infections need to be controlled in order to delay or even avoid the apparition of the symptoms (Sveger, 2017).

Protein aggregates accumulation in the hepatic tissue can produce chronic hepatopathies because of the formation of PAS-positive (Periodic Acid Schiff) polymer globules in the cells' cytoplasm (Janciauskiene et al., 2011). The development of the symptoms is bimodal, where cholestasis and neonatal cirrhosis appear before the first year of life and hepatic cirrhosis and the hepatocarcinomas (HCC) make its appearance later than the chronic obstructive pulmonary disease (COPD) symptoms normally (If COPD occurs at any stage of life). Taking into account the results of the studies performed by Hussain and collaborators (Hussain et al., 1991), it can be pointed out that from 10% to 15% of ZZ patients suffer from neonatal hepatitis, from which 5% will aggravate to a progressive cirrhosis that threatens patients live. The group that does not undergo a fibrosis can be subdivided into four different groups depending on the severity of their symptoms. S mutation produces dimmers that are not accumulated, but heteropolymers SZ might develop a disease similar to homozygous ZZ.

1.11. Neutrophils role in the disease

Neutrophils are phagocytic cells responsible for the innate immune response against bacteria and fungi. Several disease-prone conditions are directly linked to a malfunction of this cell line and up-regulation of their functions is associated with inflammatory disease (Hoenderdos et al., 2016). Intravascular neutrophils detect the focus of infection and adhere to the endothelium of capillary vessels situated in the periphery of the zone where the inflammatory process is taking place. After the adhesion, neutrophils start their migration through the blood vessel walls and interstitial tissues, where they function within adverse microenvironmental conditions (Figure 4).
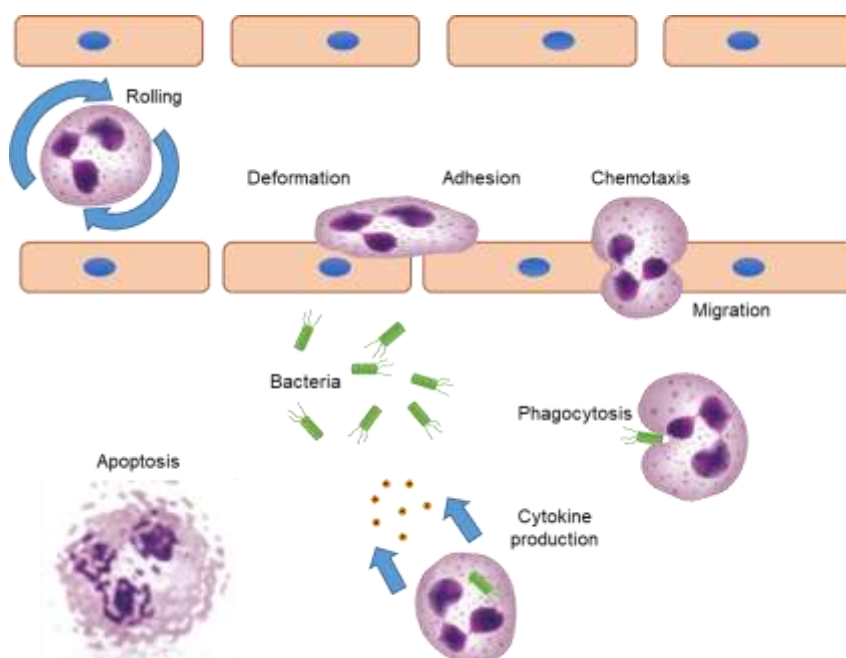

Figure 4. Mechanism of action of neutrophils as a primary response to an infection. Adapted from: (van Eeden et al., 1999)

Neutrophils exert a key effect in the pathology development; their activation when a pathogen is present in the body induces their degranulation, deploying cytotoxic compounds to the bloodstream. Those compounds, mainly proteases and reactive oxygen species (ROS), are nonspecific and react against the pathogen due to its proximity. When the protease finishes its activity is inhibited by AAT and safely secreted, but when the levels of AAT decrease, the bloodstream takes them all along the body, interacting with the host tissues (van Eeden et al., 1999).

1.12. Degranulation

Neutrophil elastase (E.C.3.4.21.37; International Union of Biochemistry and Molecular Biology (IUBMB)) is stored in the within cytoplasmic azurophilic granules and its release is triggered by pathogenic stimulation. It either acts as a free protein or is associated with neutrophilic extracellular traps (NETs) to perform its main function, which is the degradation of invading pathogens. It provides the earliest line of defense in the immune system. It is also thought to play a pivotal role in tumor invasion and metastasis as well as other inflammatory conditions (Kawabata et al., 2002).

Among the properties of elastase, we find it capable of degrading a high variety of extracellular matrix and key plasma proteins. Besides its proteolytic activity, neutrophil elastase triggers the release of interleukin-6 (IL-6) and other pro-inflammatory cytokines such as IL-8. Under physiological conditions, endogenous protease inhibitors tightly regulate neutrophil elastase's activity under inflammatory conditions, an imbalance protease/antiprotease is produced and the enzyme remains active (Kawabata et al., 2002). Upregulation of proteolytic activity of neutrophil elastase in lung and its surrounding environment leads directly and indirectly to a variety of changes related to the pathophysiology of acute lung injury.

In AATD, the imbalance protease/antiprotease is constant, where the AAT levels are lower than the normal values. The neutrophil elastase remains active for longer, causing more damage to the tissues.

1.13. Hypoxia and oxidative stress in AATD

Activated neutrophils increase their oxygen consumption, which drives into the generation of ROS using nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. ROS production contributes in decreasing the local oxygen levels with the combination of superoxide dismutase (SOD1, SOD2), Myeloperoxidase (MPO), oxygen peroxide (H_2O_2) and hypochlorous acid (HClO) creating local hypoxia and oxidative damage to the affected tissues (Williams and Chambers, 2016).

Diverse groups have manifested that oxidative damage is a factor that highly contributes to hepatic and pulmonary damage in AATD animal models, and studies in humans show that this evidence might be also a reality in humans. Prior studies carried on by our research group have demonstrated that the oxidative stress produced by an imbalance in the antioxidative defenses in pediatric asymptomatic AATD patients is related to a higher probability in developing hepatic or pulmonary disease. Patients with high (ZZ) and moderate risk (SZ) of developing emphysema showed higher oxidative damage in the DNA (8-hydroxy-2'–deoxyguanosine (8-OHdG)), in the lipids (malondialdehyde (MDA)) and in the oxidized proteins (carbonylation) with the consequent augment in the oxidative stress. Comparing with the

control group, the intermediate and high-risk groups showed a significant decrement in the total oxidized and reduced glutathione (GSH) and a low catalase activity. This reduced activity in the oxidation protectors leads to H_2O_2 accumulation, which would explain the incremented oxidative stress biomarkers in these patients. No differences were observed among the controls (MM) and low-risk groups (MS, SS). In addition, different stages of the parameters were created among the patients, as the expression of the Z allele has more severe consequences in homozygous ZZ than heterozygous (MZ, SZ) (Escribano et al., 2015).

Studies with murine models suggest that hypoxia and inflammation molecular pathways are closely related. When mice are exposed to 5% O_2 during a one-hour period, the plasma levels of pro-inflammatory cytokines rise (IL-6, tumor necrosis factor alpha (TNFα), and IL-1) (Ertel et al., 1995). In addition, other studies reported increased plasmatic concentrations of IL-6, IL-1RA (IL-1 receptor agonist) and C reactive protein in volunteers that stayed 3 days in high altitude (Hartmann et al., 2000). Inflammation in the surroundings of the pulmonary tissue is driven by the release of pro-inflammatory cytokines as IL-6 and IL-8. These interleukins mediate in the recruitment of neutrophils to the affected zone, increasing the cellular concentration and lowering the oxygen available even more, which ends up in a feedback loop of damage-inflammation-recruitment (Figure 5)(Sadik et al., 2011).
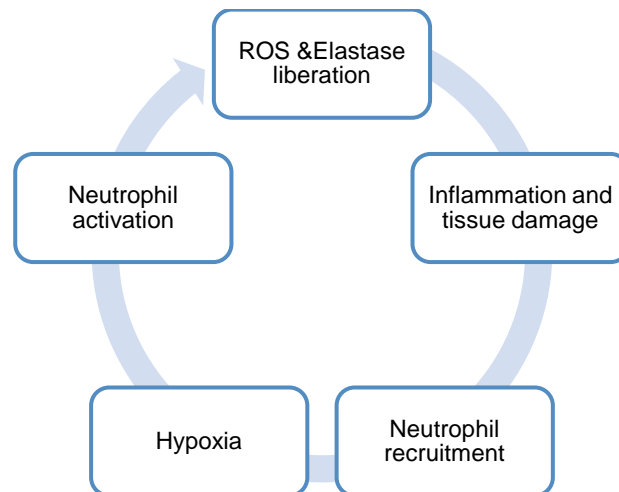
*Figure 5. Positive feedback loop triggered by the activation of the neutrophils. **ROS** = reactive oxygen species.*

Finally, neutrophils have a short lifespan in homeostatic conditions, and they normally undergo apoptosis in order to avoid the release of their granules. However, it has been demonstrated that their mean lifespan increases dramatically in hypoxia conditions, delaying the resolution of the inflammation and releasing proteinases and ROS, which ends up in tissue damage (Walmsley et al., 2005) (Figure 5).

With all the information regarding oxidative activation, the focus of this study is to determine how neutrophils activate differentially while they undergo hypoxic or normoxic conditions, and measure their oxidative stress in order to find a correlation between the factors.

2. Aims

1) Create a procedure for a long-term study in which neutrophils from AATD patients and healthy volunteers will be compared in hypoxia versus normoxia and the differences in degranulation, oxidative profile, ROS production and gene expression will be stated.

2) Observe the differences between the neutrophil degranulation, and ROS production in patients with AATD (ZZ and SZ), patients with at least one allele of the disease and healthy volunteers. Among the patients with AATD, evaluate the differences among the phenotypes. There is not data that evaluates the consequences of the intermediate plasmatic levels (MZ) on the neutrophil's physiology, which is important taking into account the high numbers of individuals with this phenotype. On the other hand, the results of previous studies show that MZ individuals have a bigger risk of developing pulmonary disease in comparison to controls MM.

3. Materials and methods

3.1. Blood samples collection

Blood samples were obtained from the pediatric department of the Hospital Clínic Universitari de València (Valencia, Spain). In total, 21 volunteers participated in this study, 5 controls and 16 patients with different phenotypes. A consent letter was presented to every patient and control explaining that all the private data from this study is confidential and the results obtained will be presented as anonymous data (see annex 1). Blood collection was not performed until having the signature of the patient or legal representative.

The inclusion criteria were: (1) patients diagnosed with DAAT, in accordance with the American thorax society (ATS), European respiratory society (ERS) and Spanish society of pneumology and thoracic surgery (SEPAR) recommendations, (2) healthy control individuals with no history or clinical findings suggesting pulmonary or hepatic disease. In both pediatric and control individuals, the following exclusion criteria were applied:

1. Cardiac dysfunction.
2. Active infection.
3. Autoimmune diseases.
4. Neurological disorders.
5. Psychiatric disorders; cancer.
6. Antioxidant treatment three months prior to taking biological sample.
7. Surgery three months prior to the biological sample taking.

Previous studies have shown that all these aspects modify the levels of oxidative stress.

Trained nurses performed the blood collection in three tubes of BD Vacutainer™ Plastic Blood Collection Tubes with K2EDTA (ethylenediaminetetraacetic acid) (3 mL) (BD Biosciences) for obtaining a total volume of ~9 mL (Figure 6). All reagents used are pyrogen-free.
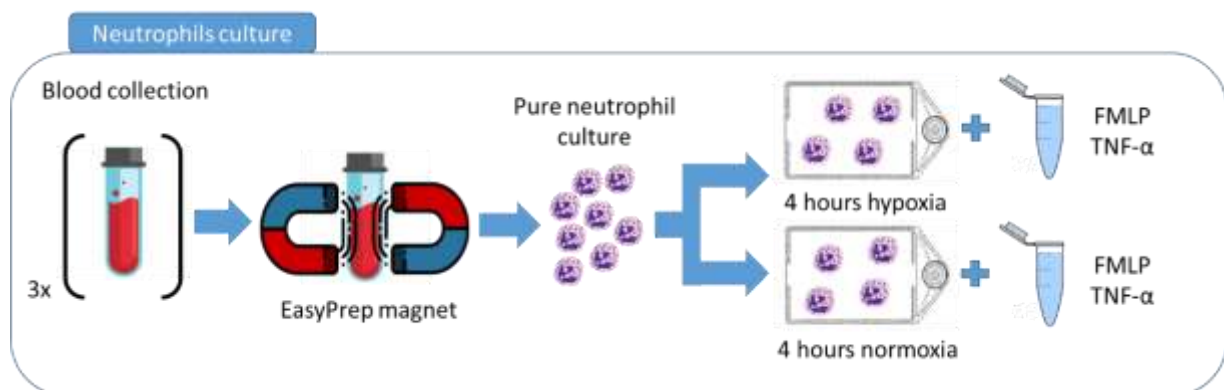
*Figure 6. Scheme about the neutrophils isolation and culture method. Three Blood collection tubes are required for the negative separation of the neutrophils using magnetic beads coated with antibodies. The pure neutrophil culture is divided into 2 T25 flasks and one is cultivated 4 hours in hypoxia and the other in normoxia. After the culture, they are stimulated with fMLP and TNFα. **fMPL** = formylated peptide; **TNFα** = tumor necrosis factor alpha*

3.2. Neutrophil isolation by negative selection

The negative selection was performed using antibody-driven magnetic beads with EasySep™ Direct Human Neutrophil Isolation Kit (StemCell); this kit contains two reactive compounds: an isolation cocktail containing monoclonal antibodies in phosphate buffered saline (PBS) and a

Fc (fragment crystallizable) receptor blocking antibody, and a suspension of magnetic particles and monoclonal antibodies in PBS.

The protocol was optimized for 9 mL samples and performed under sterile conditions. The media used for the separation was Dulbecco's Phosphate Buffered Saline (DPBS) without calcium or magnesium, to prevent neutrophil activation.

3.3. Neutrophils culture

After performing the isolation, neutrophils were divided into two different T25 flasks, one was cultured in hypoxic conditions (94% N_2, 5% CO_2, 1% O_2) for 4 h (hours) and the other one was cultured in normal culture conditions (5% CO_2, 21% O_2) 4h. Media added to the hypoxic cells was left overnight (O/N) in hypoxic conditions for facilitating the gas exchange and was carried hermetically sealed to the working area. Addition of hypoxic medium is counted as time 0.

1 mL volume of hypoxic media was added to the pellet formed, then equally distributed between the flasks and after this, the corresponding media was added to normoxic and hypoxic cells until reaching a concentration of $2 \cdot 10^5$ cells/mL. Cell counting was performed using a Neubauer improved (BrandBlau) chamber adding 1:1 of trypan blue for measuring the viability.

After 4 h, two stimulating factors were added. TNFα (20 ng/mL) was left acting for 30 min (minutes) and the formylated peptide (fMLP) 10 mM for 10 min in both conditions to see the differences in activation and degranulation. fMLP is dissolved in dimethyl sulfoxide (DMSO), which has an activation effect in neutrophils, so the concentrations of this compound were kept under 0.1% of the total volume in order to minimize this side effect.

After the isolation, culture, and activation, the sample was split in two; one used fresh for flow cytometry and the rest of the sample was frozen (separating pellet and supernatant by centrifugation at 600 g 5 min) for immunoassays and reverse transcription quantitative polymerase chain reaction (RT-qPCR) studies. Depending on the initial concentration of neutrophils, 6 to 10 1.5 mL Eppendorf tubes were used.

3.4. Lowry assay

Lowry assays are used in order to measure the total amount of protein present in each of the patients' frozen samples in a 96-well plate. 7 wells were filled with decreasing concentration (1.5, 1, 0.8, 0.6, 0.4, 0.2 and 0 mg/mL) of Bovine serum albumin (BSA) for the creation of a standard curve and different sample dilutions were added too for a correct measurement. Curve and samples were measured by duplicate

In order to start the measurement, the Biuret reaction was triggered adding an alkaline media and copper sulfate (II), which turns the solution blue. Afterward, a mixture of phosphotungstic and phosphomolybdic acid (Folin reagent) is added to the media, which in combination with the copper-treated proteins, is reduced (loses 1, 2 or 3 oxygen atoms) and produces blue reduced species with a peak absorbance at 750 nm which is afterward read in a spectrophotometer (Spectramax Plus 384, Molecular Devices).

The reaction takes part mainly due to the amino acids tyrosine and tryptophan amino acids, because of their aromatic rings but also by cystine, cysteine, and histidine. In these assays, different supernatants were diluted from 3:100 and 5:100 compared to the standard curves due to the high amount of proteins present in the media.

3.5. Assessment of neutrophil degranulation

Elastase degranulation

Elastase degranulation was measured using Elastase degranulation kit (Cayman). In this kit, a specific non-florescent elastase substrate (Z-Ala-Ala-Ala-Ala)2Rh110 is used to measure the elastase activity. If active, elastase cleaves the alanine residues and releases de highly fluorescent compound R110. This compound is excited at 485 nm and its emission reaches a peak at 525 nm. It can be measured with a fluorimeter later on. In order to perform the assay, black plates included in the kit were used.

The plate is divided into 2 parts: standard wells and sample wells. The standard wells are filled with decreasing concentrations of human elastase (10 U/mL, 5 U/mL, 2.5 U/mL, 1.25 U/mL, 0.625 U/mL, 0.313 U/mL, 0.156 U/mL) and a blank. Which allows the creation of a standard curve, where the slope is used for calculating the enzyme concentration in the sample wells.

For the sample wells, 10 µL of defrosted culture supernatant from each well of the experimental plate have to be transferred to a corresponding well on the plate. Then 90 µL of the diluted assay buffer is added to the sample wells. The final volume for each sample well before addition of the substrate should be the same as that for the standard wells (100 µL).

When the set up is complete, 10 µL of the substrate (Z-Ala-Ala-Ala-Ala)2Rh110 have to be added for the reaction to begin, and after an incubation of 1.5 h at 37°C, the plate is read in a fluorimeter (SpectraMax Gemini XPS, Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. All measurements were performed in duplicate.

Myeloperoxidase Activity

MPO (EC 1.11.1.7) is the most expressed protein in azurophilic granules in neutrophils. When MPO reacts with the H_2O_2 present, forms intermediates MPO-I and MPO-II that oxidize Amplex® Ultrared reagent from the myeloperoxidase activity assay kit (EnzChek) and create a fluorescent product that can be detected. By using this kit, we aim to measure the quantity of MPO present and active in the samples.

Standards were prepared using a descendant concentration of MPO starting from 200 ng/mL and halving it until reaching 1.5 ng/mL. In this particular experiment, 50 µL of defrosted supernatant was added to a 96-well microplate followed by the addition of 50 µL of a 2X solution and a 30 min incubation. The fluorescence is then read at 590 nm after an excitation at 530 nm (SpectraMax Gemini XPS, Molecular Devices). All measurements were performed in duplicate.

Human Lactoferrin

Lactoferrin is released during inflammatory processes. This transferrin has different roles in the organism as it has been reported to have antibacterial, antiviral, anti-inflammatory and immunoregulatory properties.

Human lactoferrin enzyme-linked immunosorbent assay (ELISA) kit (Abcam) detects minimum amounts of lactoferrin present in the supernatant of neutrophil's culture by the ELISA sandwich technique. The signal from the secondary antibody can be detected by a colorimetric assay. To perform the assay, 1:500 dilution of 50 µL of defrosted neutrophils supernatant from the patients and the controls were added to a 96 wells plate containing the primary antibody. After 1h incubation, it was washed 3 times and 50 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was used for triggering the reaction. After 10 min, 50 µL stop solution (HCl) was

added and the optical density (OD) read at 450 nm (Spectramax Plus 384, Molecular Devices). All measurements were performed in duplicate.

3.6. Oxidative stress measurements by flow cytometry

The technology of the flow cytometry has provided a rapid and reliable way of acquiring data related to the oxidative stress. Several fluorophores allow the detection of the main ROS and the damage they can do to compounds and organelles. Initial trials of viability and purity were tested using BD FACSVerse Flow Cytometer (BD Bioscience); and the extraction of the data from the patient's culture was performed using two different flow cytometers: LSRFortessa® X20 Flow cytometer (BD Bioscience) and a FACSARIA® III Cell Sorter (BD Bioscience) analyzed the pure neutrophils culture obtained each day.
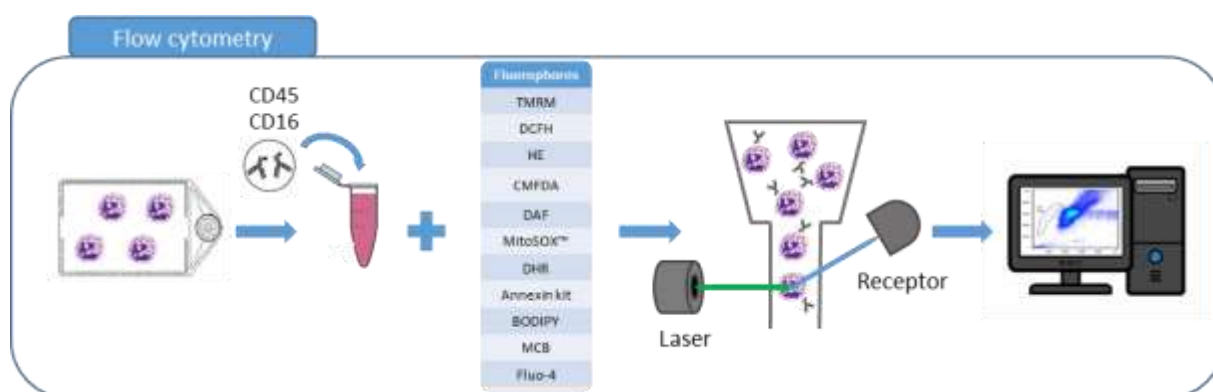

Figure 7. Scheme about the Flow cytometry procedure. Fresh culture is stained with anti CD45 and anti CD16 for testing neutrophils. Fluorophores are then applied and, after incubation, they pass through the flow cytometer. **BODIPY** = *4,4-difluoro-4-bora-3a,4a-diaza-s-indacene;* **CMFDA** = *5-chloromethylfluorescein diacetate;* **DAF** = *4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate;* **DCFH** = *2' 7-Dichlorohydrofluorescein;* **DHR** = *1,2,3 – dihidrorhodamine;* **FTC** = *Fluorescein-5-thiosemicarbazide;* **HE =** *Hydroethidine;* **MCB** = *Monochlorobimane;* **TMRM** = *Tetramethylrhodamine methyl ester perchlorate.*

Neutrophils detection is performed with two markers: CD45 (lymphocytes-specific), and CD16 (neutrophils-specific) antibodies. The total number of cells per assay and fluorophore was 20,000 on a total volume of 200 µL of media (Table 2).

Detectors
In the cytometer, two parameters are typically obtained: light scattering and fluorescence.

Light scattering: Light changes its direction because of an interaction with an object, in this case, cells and cell particles. Flow cytometers measure light scattering in two directions depending on the angle formed with the light source: forward (180º) (FSC) and side (90º) (SSC). The light reflection allows a correlative measurement of the size and the complexity (membrane roughness and granularity) of the cell respectively.

Fluorescence: Fluorophores are molecules that can be excited by specific wavelengths and then emit the light back after an energy loss. Specificity of excitation and emission is critical for the identification of the compound and allows the distinction on the measurement of a specific particle. The fluorophores used for the measurement are presented in the following table (Table 2.)

Table 2. Fluorophores used for the measurement of the parameters of the oxidative stress present in the cell. The parameter to be measured is shown in the left, the compound used for the measurement, the laser channel used in the cytometer (pre-set in the cytometer software) and the voltages used for the detection.

Parameter	Volume and concentration of use	Channel Measurement	Voltages FITC/PE/PECy7/BV-421
ONOO⁻ (ROS)	1 µL DHR (50 µg/mL)	FITC	308/-/-/-
Intracellular Ca²⁺	2.5 µl Fluo-4 (50 µM)	FITC	220/-/-/-
GSH	0.63 µL MCB	FITC	-/-/-/368
Membrane potential	0.63 µl TMRM (240 µM)	PE	190/192/505/313
H₂O₂	0.63 µl DCFH (1 mg/mL)	FITC	190/192/505/313
O₂⁻	0.63 µl HE (1 mg/mL)	7AAD	308/291/505/313
Thiols	0.63 µl CMFDA (10 µM)	FITC	308/291/505/313
NO	0.20 µl DAF (1.25 mM)	FITC	308/291/505/313
Mitochondrial O₂	0.32 µl MitoSOX™ (0.5 mM)	7AAD	-/346/505/-
Carbonylated proteins	0.20 µl FTC (1 mM)	FITC	308/291/505/313
Lipid peroxidation	0.20 µl BODIPY (1 mM)	PE/APC	
Apoptosis	Annexin kit	FITC	280/450/505/-

Annexin kit = *annexin V-FITC Apoptosis Staining / Detection Kit;* ***BODIPY*** = *4,4-difluoro-4-bora-3a,4a-diaza-s-indacene;* ***CMFDA*** = *5-chloromethylfluorescein diacetate;* ***DAF*** = *4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate;* ***DCF*** = *2' 7-Dichlorohydrofluorescein;* ***DHR*** = *1,2,3 – Dihydrorhodamine;* ***FTC*** = *fluorescein-5-thiosemicarbazide;* ***H₂O₂*** = *hydrogen peroxide;* ***HE*** = *Hydroethidine;* ***MCB*** = *Monochlorobimane;* ***NO*** = *nitric oxide;* ***O₂⁻*** = *superoxide anion;* ***ONOO⁻*** = *peroxynitrites;* ***GSH*** = *reduced glutathione;* ***ROS*** = *reactive oxygen species;* ***TMRM*** = *Tetramethylrhodamine methyl ester perchlorate;* ***Δψm*** = *mitochondrial membrane potential.*

Hydroethidine (HE)

Dihydroethidium, also called HE is used as a superoxide indicator. Its blue fluorescence is present in the cytosol until oxidized to 2-hydroxyethidium by superoxide anion (O_2^-). It is capable of intercalating within the cell's DNA and stain its nucleus. HE absorbs at $\lambda ex = 358$ nm and emits fluorescence at $\lambda em = 461$ nm, a fact that allows detecting the presence of superoxide anion.

5-chloromethylfluorescein diacetate (CMFDA)

CMFDA shows the total levels of free intracellular thiols in different cell lines. Its emission reaches its maxima at $\lambda ex = 492$, $\lambda em = 517$ nm (Barrera et al., 2017).

Tetramethylrhodamine methyl ester perchlorate (TMRM)

TMRM is the fluorochrome used for measuring the membrane potential ($\Delta\psi_m$) between the inner and the outer part of the mitochondria. TMRM accumulates in functioning mitochondria that can maintain $\Delta\psi_m$, it is excited at a maximum wavelength of excitation (λex) = 548 nm and shows a bright signal with an emission peak at a maximum wavelength of emission (λem) = 574 nm. If $\Delta\psi_m$ is lost, TMRM accumulation ceases and the signal fades or completely disappears. This signal is detected by the flow cytometer, allowing detection of ROS damage present in mitochondria.

2' 7-Dichlorohydrofluorescein (DCFH)

For the observation of ROS, the fluorochrome used is DCFH. It is a cell-permeable fluorophore that is oxidized when H_2O_2 or other electron acceptors are present. After the intracellular esterases dissociate the acetate groups from the molecule, it is transformed into 2',7'-dichlorofluorescein, very fluorescent and detectable by the flow cytometer. λex = 495 nm, λem = 529 nm.

Monochlorobimane (MCB)

MCB is a nonfluorescent probe until it is conjugated with low molecular weight thiols. Among those thiols, we can find GSH, N-acetylcysteine, mercaptopurine, peptides and plasma thiols. Unlike the CMFDA, The glutathione conjugate can be distinguished from others as it has absorption/emission maxima λex = 394 nm λem = 490 nm.

4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM)

DAF-FM is a highly precise reagent used for the detection and quantification of low concentrations of nitric oxide (NO). Its reaction with NO causes a change of his chemical structure to a fluorescent benzotriazole, that is excited at λex = 495 nm and its emission can be detected at λem = 515 nm.

MitoSOX™

Mitochondrial superoxide can be visualized using MitoSOX™. The superoxide present, either by the ROS generation systems or the reactive nitrogen species (RNS), rapidly oxidize MitoSOX™, creating a product that becomes fluorescent when bound to the nucleic acids. It is generally used for the discovery of agents that modulate the oxidative stress in diverse pathologies, as it directly measures the superoxides produced in living cell's mitochondria.

1,2,3 - Dihydrorhodamine (DHR)

DHR is a chromogen capable of trespassing the cell membrane, where it is oxidized to cationic rhodamine by peroxynitrites ($ONOO^-$) produced as a subproduct of respiration. It is the most used chromogen for diagnosis and one of the most sensitives λex = 500 nm, λem = 536 nm.

Fluo-4

Calcium indicators are molecules that increase their fluorescence when bound to Ca^{2+}; Fluo-4 is used in flow cytometry studies in order to detect calcium signaling across the cell. It is excited at λex = 488 nm and its emission peak is found at λem = 520 nm.

Annexin V-FITC Apoptosis Staining / Detection Kit

This kit is based on the apoptosis studies that revealed a translocation of the membrane phosphatidylserine (PS) from the inner to the outer face of the plasma membrane. Once on the cell surface, PS is detectable by a fluorescent conjugate of Annexin V, which shows a high affinity for PS. Propidium iodide (PI) is also used in this kit in order to perform an apoptosis vs necrosis assay, as PI is permeable to dead cells. λex = 488 nm, λem = 530 nm.

4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)

BODIPY is a fluorophore created in 2009 useful for marking the oxidized lipids in flow cytometry. Among its characteristics, it is highlighted a uniquely small Stokes shift (difference between excitation and emission wavelengths) λex = 503 nm, λem = 512 nm and highly hydrophobic properties.

Detection of carboxylic acids of proteins is performed with this fluorophore due to its reactivity and liberation of fluorescein, detectable by the cytometer, its excitation is performed at $\lambda ex = 490$ nm and its emission reaches a peak at $\lambda ex = 525$ nm.

Inductors

As a positive control in the flow cytometry assay, the reactive species have to be formed by molecules also called inductors and detected with the fluorophore at the same voltage than the basal measurement. Each inductor is specific for the creation of a particular marker of the oxidation. (Figure 3.)

Table 3. Inductors used for the production of a particular element for ROS detection. Time column shows how much time was the inductor left in the well before adding the fluorophore. "Kinetic" means that is added while measuring in the cytometer. Concentration shows the concentration of use.

Inductor	Parameter	Time	Concentration
PB	O_2^-	15 min/Kinetic	1 mg/mL
NOR-1	NO	Kinetic	1 mg/mL
DEM	GSH	1 h	1 mM
FCCP	$\Delta\psi_m$	15 min	10 mM
Tert-Butyl	H_2O_2	15 min	7.7 mM

PB = plumbagin, 5-Hydroxy-2-methyl-1,4-naphthoquinone; **NOR-1** = (+/-)-(E)-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide; **DEM** = Diethyl Maleate; **FCCP** = Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; **Tert-Butyl** = Tert-Butyl hydroxide.

5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin; PB)

PB is generally classified as a toxin extracted from the carnivorous plant family *Plumbaginaceae*. It has been found to have different utilities in pharmacology and biochemistry as anticoagulant, contraceptive, adjuvant, and carcinogen agent. Its toxicity remains on the topoisomerase II arrest by H_2O_2 generation, producing double strand DNA brakes and, for this reason; it is widely used as an inductor for intracellular H_2O_2 production (Kawiak et al., 2007).

(+/-)-(E)-Methyl-2-[(E)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide (NOR-1)

A nitric oxide donor and cysteine protease inhibitor. The exact mechanisms of NO formation still need to be elucidated, but it is supposed than inter, or intramolecular electron transfer occurring during the spontaneous decomposition of this molecule is the key for the NO formation. Its application in flow cytometry has proven to increase intracellular NO and trigger the cellular response to it making it suitable as a control.

Diethylmaleate (DEM)

DEM has several regulating roles in the cell when added to the media. It has been reported that inhibits the mitochondrial aldehyde dehydrogenase and acetaldehyde oxidation by depleting glutathione.

Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)

FCCP is a photophore that stops the mitochondrial membrane potential by stopping the proton gradient formed in the mitochondrial inner membrane. Applying FCCP in flow cytometry shows a reduction in the PE measurements of samples dyed with TMRM as the $\Delta\psi_m$ is disrupted.

Tert – butyl hydroperoxide

Tert-butyl hydroperoxide has a myriad of applications including oxidative stress-inducing agent, polymerization agent and an agent for inducing peroxidation. This is the reason why is used for inducing lipid peroxidation, oxidizing thiols, and H_2O_2 production.

Basal measurements of Fluo-4, MCB, MitoSOX™, DHR, HE, CMF, DAF, FTC, TMRM, and DCF are performed in 96-well plates with pure neutrophil culture ($2 \cdot 10^5$ cell/mL) at the concentration of use shown in Table 2. HE/CMF and TMRM/DCF are placed in the same well, as their emission peaks do not overlap. Firstly, they are incubated for 20 min at 37ºC in hypoxia or normoxia depending on when the sample was cultured initially. Induced measurements for MitoSOX™, HE, CMF, TMRM, and DCF are also performed in a 96-well plate, inducing first the 200 µL of pure neutrophil culture ($2 \cdot 10^5$ cell/mL) 15 min at 37ºC with the appropriate inductor (Table 3) and condition (hypoxia/normoxia). After the induction finishes, samples are incubated 20 min at 37ºC in hypoxia/normoxia, depending on when the sample was initially cultured.

All samples are dyed with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) at 100X concentration 5 min before analyzing with the cytometer (in order to differentiate living cells from dying cells measured with the channel BV421). The only exceptions were MCB and the Annexin kit, where PI 100X is added (measured with the channel PE-Cy7) due to the overlap of the fluorophore emission with the DAPI emission. Data is obtained with LSRFortessa® X20 Flow cytometer (BD). For the measurement of the oxidized lipids, BODIPY is added to the cells and incubated 30 min in a test tube. Induced measurements of BODIPY are performed with tert-butyl, adding 5 mg/mL 20 min and then incubating 30 min with BODIPY. The cytometer used is the FACSARIA® III Cell Sorter (BD Bioscience).

For obtaining the induced measurement of MCB and DHR, a kinetic induction is performed. MCB and DHR are added to a test tube containing the cell samples and are incubated 15 min 37ºC in hypoxia or normoxia. When staining is completed, 1000 events are measured in the flow cytometer (BD Bioscience). Test tube is removed and NOR-1 (1 mg/ mL) or PB (1 mg/ mL) and NOR-1 (1mg/ mL) respectively are added. Finally, the test tube is placed again in the cytometer and the induction curve is observed.

3.7. RT-qPCR

RT-qPCR is a widely used method for gene expression quantification. In this particular experiment, RNA, DNA, and proteins were extracted with the Nucleospin Triprep 50 DNA, RNA and Protein purification kit (Macherey-Nagel) following the manufacturer's protocol. DNA and protein were kept frozen at -20ºC and -80ºC respectively for later experiments.

The proceedings start with the mRNA extracted from the neutrophils samples. mRNA is always kept in ice except when manipulated. The first step was to measure the RNA with a spectrophotometer Nanodrop 2000®, (Thermo Scientific) and adjust the samples to 2 ng/µL for the RT-PCR (Figure 8.).

The next step involved the reverse transcription; in this case, the reagents used were from the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 20 ng in a volume of 10 µL of RNA were reverse-transcribed into complementary DNA (cDNA) following the standard procedure. The X2 master mix was prepared: 10X RT Buffer 2.0 µL 25X dNTP Mix (100 mM) 0.8 µL 10X RT Random Primers 2.0 µL MultiScribe™ Reverse Transcriptase 1.0 µL 1.0 µL Nuclease-free H2O 4.2 µL, meaning a total of 10 µL to be mixed with the 10 µL RNA solution.
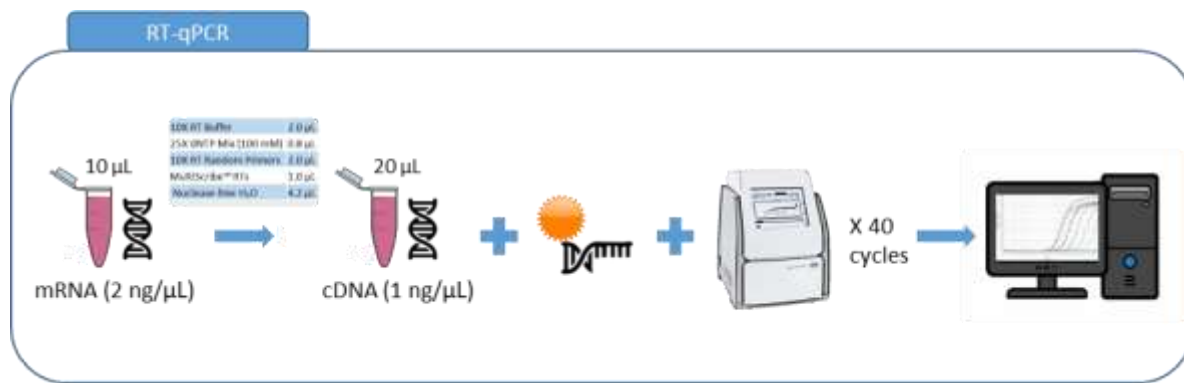
*Figure 8. Scheme about the reverse transcription - quantitative polymerase chain reaction (RT-qPCR) procedure. mRNA is transformed into cDNA using the RT reagents. Afterward, a fluorescent-tagged primer for the gene of interest is added, and polymerase is used to amplify the cDNA. Each time the primer is used, it liberates the fluorescent tag, allowing to quantify the amount of DNA present **cDNA** = complementary DNA; **dNTP** = deoxynucleoside triphosphate; **mRNA** = messenger RNA; **RT** = Reverse transcription; **RTs** = reverse transcriptase.*

The final volume was 20 µL, reaching a final concentration of 1 ng/µL. Following repetitions of the experiment were run adjusting the reagents in order to obtain always the same final concentration of 1 ng/µL. The RT-PCR protocol is performed using the thermocycler Applied Biosystems 9800 Fast Thermal Cycler (Applied Biosystems) following a four-step cycle of 25ºC, 10 min, 37ºC 120 min, 85ºC 5 min and 4ºC O/N.

TaqMan™ Fast Universal PCR Master Mix (2X) (TaqMan) was used for the quantitative PCR (qPCR). The gene probes used for the qPCR protocol and the function of the targeted genes (mainly related to oxidative stress, activation, degranulation, and viability) are shown in Table 3. *GADPH* act as housekeeping genes as it has been proved a good expression marker.

Table 4. Housekeeping and target genes amplified using the qPCR protocol.

Target Name	Codified gene	Function
ARNT	Aryl hydrocarbon receptor nuclear translocator	Hypoxia responses
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like	Hypoxia responses
CAT	Catalase	Antioxidant responses
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism
GCLC	Glutamate-cysteine ligase catalytic subunit	Antioxidant responses, glutathione synthesis
GCLM	Glutamate-cysteine ligase modifier subunit	Antioxidant responses, glutathione synthesis
GPX	Glutathione peroxidase	Antioxidant responses
GSR	Glutathione-disulfide reductase	Antioxidant responses
HIF1A	Hypoxia-inducible factor 1α	Hypoxia responses
hTERT	Telomerase	Apoptosis regulator
NFE2L2	Nuclear factor, erythroid 2 like 2	Gene Activation
SOD1	Cu/Zn superoxide dismutase	Antioxidant responses
SOD2	Mn superoxide dismutase	Antioxidant responses

Genes under analysis are related to the oxidative responses, thus, they are genes of interest for this study. The components used in the qPCR are provided by TaqMan™ Fast Universal PCR Master Mix (2X) kit (TaqMan), used in a 384-well plate following the manufacturer's instructions. Each well contained 0.7 ng DNA, 0.4 µL probe, 5 µL TaqMan™ Gene Expression Master Mix (TaqMan) and 3.9 µL de nuclease-free water. Samples were done by duplicate. A 4 step program was used: (1) 50 ºC 2 min, (2) 95 ºC 10 min, (3) 40 PCR cycles 95ºC 15 s and 60 ºC 1 min, and (4) 25 ºC ∞ using the thermocycler QuantStudio® 5 (Applied Biosystems).

In the qPCR, a positive reaction is detected by the accumulation of a positive fluorescent signal. The cycle threshold (Ct) is defined as the required number of cycles for the fluorescent signal to surpass a threshold (normally exceed the basal values). Ct values are inversely proportional to the quantity of nucleic acid present in the sample. Previous studies show Ct values < 29 are very positive and indicate high expression of the gene of interest. Cts with values 30-37 are positive and indicate moderate expression of the aimed gene. Higher Cts normally represent contamination of the wells.

For the results comparison, the delta-delta Ct (ΔΔCt) method was used. This method compares the unknown sample with a calibrator gene (in this case *GADPH* and *GUSB*). The ΔΔCt value determinates the difference of expression between those genes and is obtained with the formula (Livak and Schmittgen, 2001):

$$Quantity\ of\ RNA =\ 2^{-(\Delta\Delta Ct)}$$

Equation 1. Delta-delta Ct (ΔΔCt) method where ΔΔCt equals the difference among the differences of the mean value of the gene of interest against the mean value of the calibrator gene and the value of the gene of interest minus the calibrator gene for each sample. ΔΔCt = (Mean interest gene – Mean calibrator gene)-(Interest gene – calibrator gene).

3.8. Data analysis

Demographic data were analyzed using the Kruskal-Wallis test. Lowry and ELISA data are expressed as the mean ± standard error of the mean (SEM) (minimum and maximum values). Total protein content, functional elastase, and lactoferrin liberated to the media values are presented in µg/mL, MPO is measured in pg/mL. Assessment of normality was performed by the Shapiro–Wilk normality test. mRNA expression of antioxidant enzymes and apoptotic proteins data followed a normal distribution and differences were assessed using ANOVA. Multiple hypothesis testing was performed (Holm–Sidak and Dunn's multiple comparisons tests) to identify the significant pairwise differences among groups. Statistical analyses were performed using the GraphPad Prism 7.00 Software (GraphPad).

Data obtained from the flow cytometers were analyzed using FlowJo® 10.0.7 (LLC) using the following procedure (Figure 9): Firstly, the region of the dot plot containing the neutrophils was selected using the parameters of FSC and SSC from the total events ($1\cdot10^4$ registered events). After discarding the events not suiting the size and morphology restrictions, the negative events for the channel BV-421 or PE-Cy7 (DAPI and PI respectively) were selected, leaving the stained neutrophils, which are assumed dead. Within this selection of living cells, the mean of the fluorescence on the different channels depending on the fluorophore (Table 2) is obtained in fluorescent units (FU), indicative of the total quantity of the interest molecule present. FU means were exported to Prism 7.00. Biomarkers of oxidative stress are expressed as average ± SEM. qPCR fold change is expressed as mean ± Standard Deviation (SD).
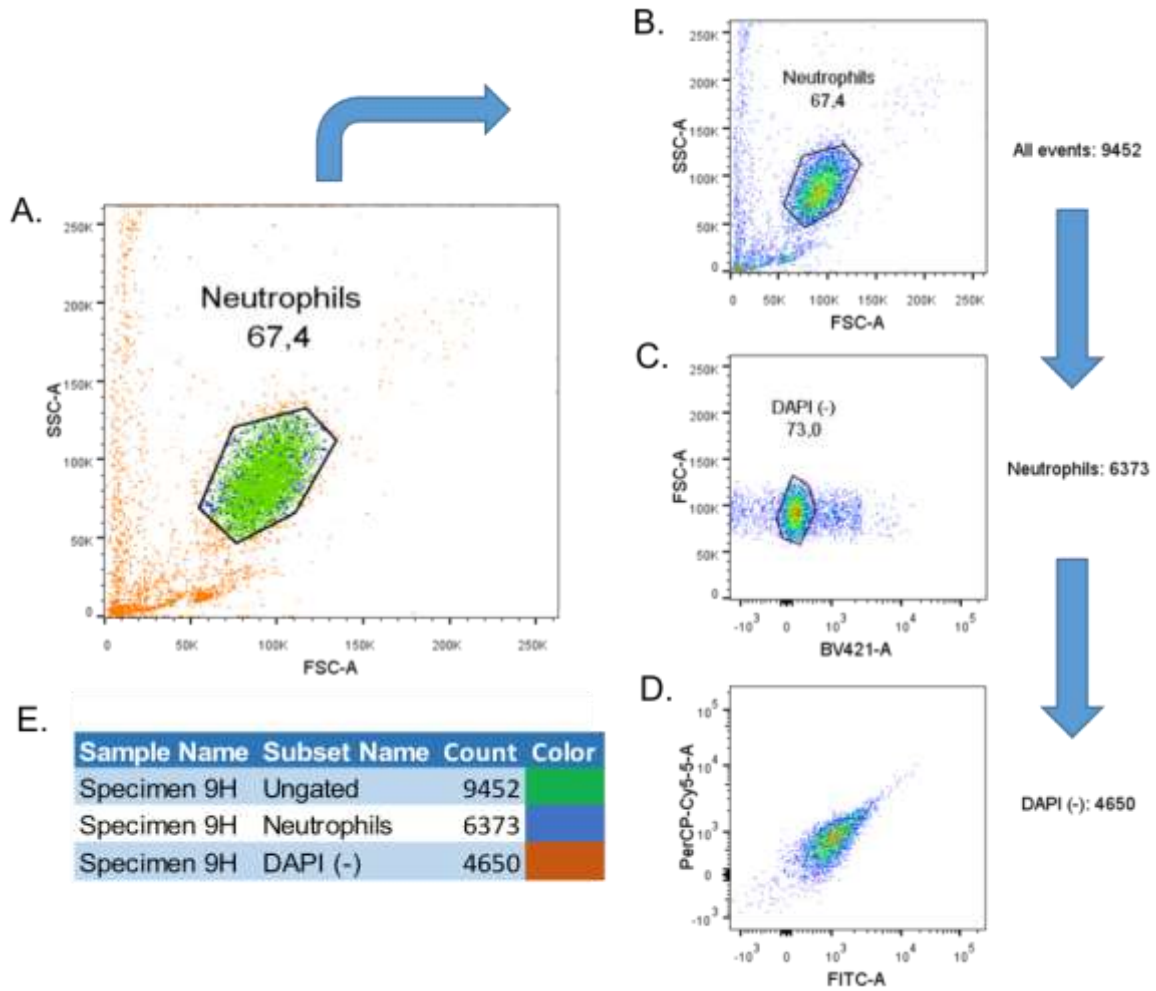
Figure 9. Method of selection of neutrophils from the events registered in the cytometer. The main image (A) shows all the separate events in the same dot plot, it is created with the combination of the following images. From the first dot plot, the events with the size and morphology similar to neutrophils are selected (B). The second dot plot is for the discrimination of the DAPI-Positive events (C), which are assumed dead. In the third dot plot, the Fluorescence is plotted and analyzed (D). Information is shown in the table (E). **DAPI** *= 4',6-Diamidino-2-phenylindole dihydrochloride;* **FSC** *= forward scatter;* **SSC =** *side scatter.*

4. Results

4.1. Demographic data

Blood from 21 volunteers (16 patients and 5 controls) was obtained from the pediatric department in the Hospital Clínic de València (Valencia, Spain). Those patients were selected accordingly to the inclusion/exclusion criteria previously defined (see section 3.1).

Patients were categorized according to their AAT phenotype in 5 different groups. A control group of 5 healthy volunteers with the MM phenotype (23.8% of the total) was also included in the study. 7 had MZ phenotype (33.3%), 3 had ZZ phenotype (14.3%), 5 SZ phenotype (23.8%) and 1 subject was SMattawa (4.8%). All the subjects were healthy according to their physical status and their pulmonary and liver tests.

Table 5. Demographics and clinical characteristics in α1-antitrypsin deficiency patients and controls.

Variable	Control	MZ	ZZ	SZ	SMattawa	P-Value
Subjects	5	7	3	5	1	
Age years	26 (9-46)	8.42 (4-11)	19.33 (13-25)	10.80 (8-16)	7	**0.0283**
Male/female	3(60%) / 2(40%)	3(42.9%) / 4(57.1%)	2(66.7%) / 1(33.3%)	3(60%) / 2(40%)	0(0%) / 1(100%)	0.4280
AAT mg·dL⁻¹#	141 (119-213)	90.3 (69-141)	28 (24-32)	71.6 (52-112)	54	**0.0003**
BMI kg·m⁻²	23 (14-26)	17.4 (15.1-21.9)	22.2 (21.9-22.5)	18 (15.5-22.8)	15,9	0.2085
FVC % pred¶	99 (91-107)	97.8 (91.5-100.7)	95.2 (89-101.4)	108.6 (94.8-120.3)	109,7	0.1280
FEV₁ % pred¶	96 (88-113)	98.2 (88.9-108.6)	98 (92-103.9)	109.3 (102-120.6)	108,5	0.1149
FEV₁/FVC% pred⁺	92 (78-114)	88.8 (79-100)	93.8 (89.5-98)	91.3 (84.4-100)	89	0.7816
AST U·L⁻¹ƒ	28 (21-37)	31.8 (22-38)	24 (19-29)	33.2 (17-47)	25	0.5722
ALT U·L⁻¹ ƒ	20 (10-40)	16.7 (12-19)	25 (19-31)	22 (14-30)	12	0.2942
GGT U·L⁻¹##	15 (12-18)	14.8 (12-20)	17 (13-21)	14.6 (11-19)	10	0.5157

Data are presented as median (range), unless otherwise stated. Bold indicates statistical significance (p<0.05) after application of the Kruskal–Wallis for not normal data (according to Shapiro–Wilk normality test) or ANOVA test if the normality test was positive. AAT = α1-antitrypsin; BMI = body mass index; FEV1 = forced expiratory volume in 1s; FVC = forced vital capacity; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = γ-glutamyl-transferase. #: normal range 90-200 mg·dL⁻¹; ¶: normal⩾80% predicted; ⁺: normal >80% predicted; ƒ: normal range 1-37 U·L⁻¹; ##: normal range 1-55 U·L⁻¹

Significant differences were found in the age of the participants regarding the controls (p = 0.028). Regarding liver damage markers, there were no differences in AST, ALT and GGT levels between groups. There were no differences in BMI and lung function tests across the groups. As expected, significant differences were observed in the AAT levels between groups. (p=0.0003). As it was explained in the introduction, AAT levels are directly related with the mutation carried by the patient (see section 1.6) (Table 5).

4.2. Optimization of the isolation of the neutrophils

Different concentrations of isolation cocktail and magnetic beads were used in order to obtain the best correlation between neutrophils purity and volume of reagents. Using 350 µL of each reactive and after that, the staining with antibodies CD45 and CD16, the FACSVerse flow cytometer showed an 85.74% of the total events within the size and form of the neutrophils using the forward and side scattering, 99.66% of those were double $CD45^+CD16^+$, confirming their lineage (Figure 10). Taking into account that the normal values for absolute neutrophil

count are between 1500 to 8000/ µL of blood, this means from 1.3 to 7.2 million neutrophils after performing the isolation protocol from 9 mL of blood sample. Counts using the Neubauer chamber shown 6 million neutrophils per patient on average, with the purity shown in Figure 10 (> 99%).

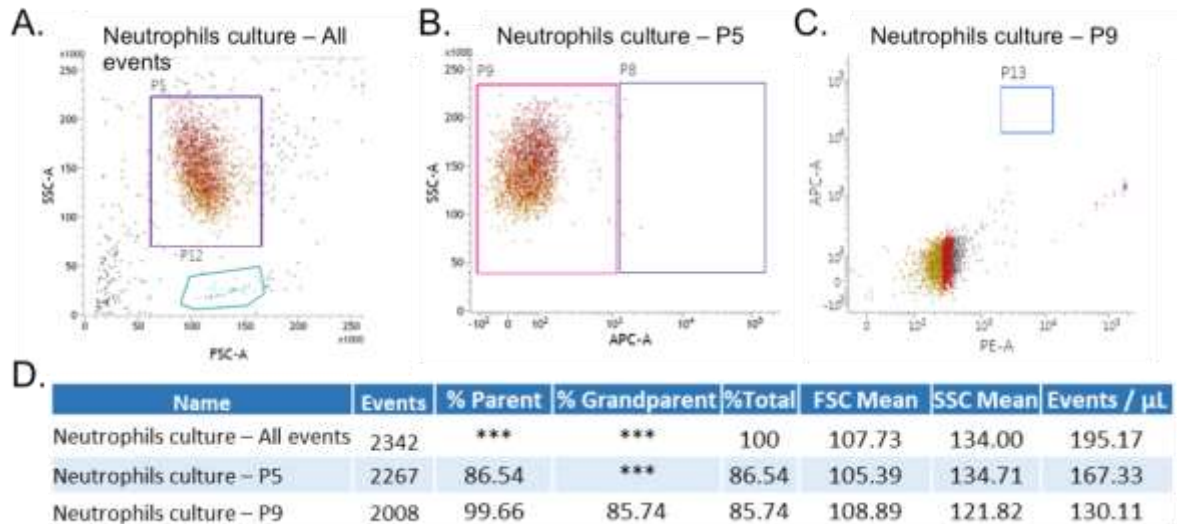| Name	Events	% Parent	% Grandparent	%Total	FSC Mean	SSC Mean	Events / µL
Neutrophils culture – All events	2342	***	***	100	107.73	134.00	195.17
Neutrophils culture – P5	2267	86.54	***	86.54	105.39	134.71	167.33
Neutrophils culture – P9	2008	99.66	85.74	85.74	108.89	121.82	130.11

*Figure 10. Purified neutrophils culture measurement by flow cytometry. The total events are shown in (A), where P5 are neutrophils, P12 share the shape and morphology of other granulocyte populations. (B) Separation of cells into positive and negative to DAPI (P8 and P9 respectively). Among the P9 population, the highly positive to APC and PE are discarded as might be artifacts (P13). Information is shown in the table (D) with the percentages of each population (D) **FSC** = forward scatter; **P** = Population; **SSC =** side scatter.*

Trypan blue staining shows viability is similar at t =0, t = 4h and t = 8h, which is the total length of the procedure. As it can be observed (Figure 11), few stained cells are present in the images (marked in orange). Photographs were taken using a microscope Eclipse Ts2 (Nikon) adapted with a camera U3CMOS (Microscopiadigital).
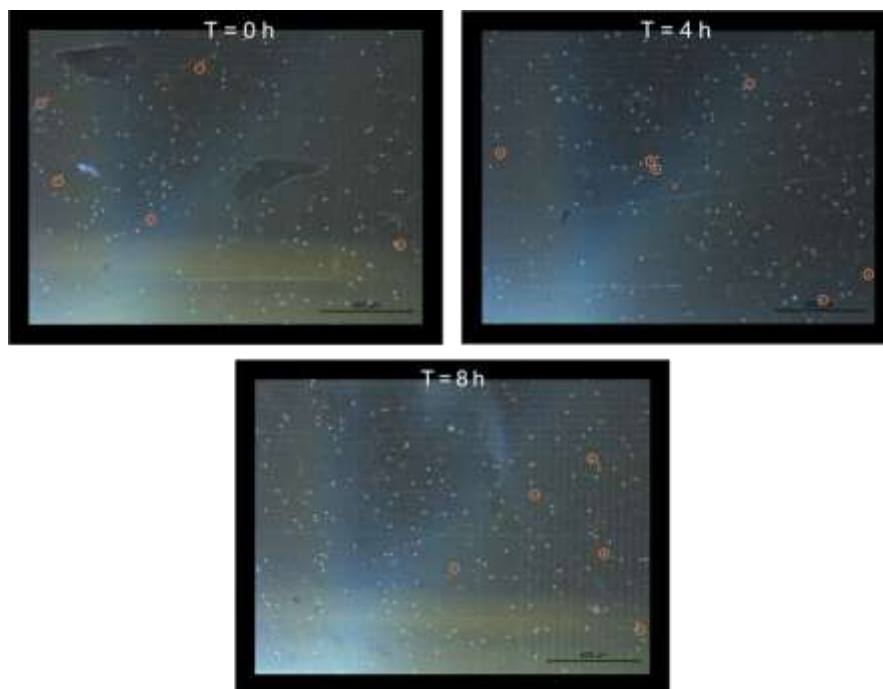

Figure 11. Trypan blue analysis of the neutrophils viability. Blue dyed cells are marked in orange and considered dead. T = 0h: 1/53, T = 4h: 3/40, T = 8h: 1/56.

4.3. Lowry assays

Data obtained from Lowry assays were normalized subtracting the measurement using wells containing hypoxia and normoxia medium as blanks. There is a significantly higher concentration of proteins in the cellular media that have undergone hypoxic conditions (P-value = 0.003) (Figure 12).
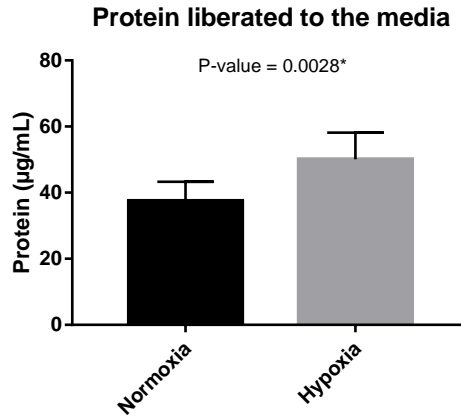
Protein liberated to the media

*Figure 12. Liberation of the protein to the media. Culture conditions affect the protein liberation to the media. 4 hours of hypoxic culture upregulates the liberation of protein to the media compared to 4 hours of normoxic culture (P-value = 0.0028). * indicates statistical difference.*

4.4. Degranulation results

No significant differences are shown between normoxia and hypoxia (P-value ANOVA = 0.5442). When samples were separated by phenotype, we observed similar values between normoxia and hypoxia conditions in MM, MZ, and SMattawa, but a trend is observed in ZZ patients, where the concentration of elastase is diminished in hypoxia and in SZ, where it is augmented, although the results did not reach statistical significance (P-value ZZ = 0,2516, P-value SZ = 0,4459). When comparing the total expression, SZ and ZZ show an increased expression in hypoxia and normoxia against the MM, however, not in a significant manner. SMattawa shows a very similar liberation to MM controls (Figure 13).
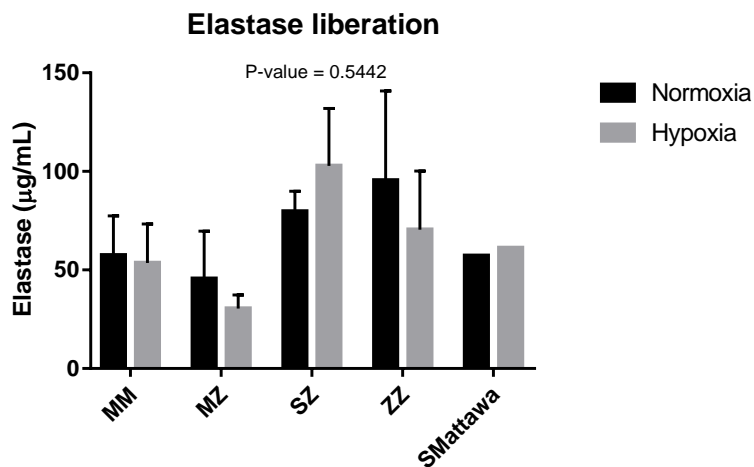
Elastase liberation

Figure 13. Liberation of elastase to the media in normoxia and hypoxia. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1.

When the results of MPO were separated into phenotypes, it was observed that MM and ZZ shared a similar distribution where the concentration in hypoxia is slightly decreased compared to normoxia. MZ and SZ show an increment in the release of MPO in hypoxia being SZ the most different compared to the controls (P-value SZ = 0.3664). In terms of total liberation, MZ and SZ liberation is higher compared to the controls. SMattawa shows a very low liberation of MPO (Figure 14).
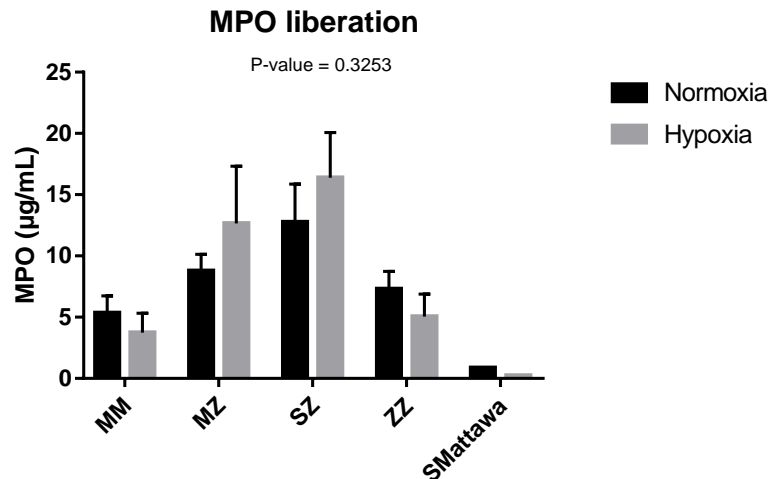

*Figure 14. Liberation of MPO to the media in normoxia and hypoxia. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1. **MPO** = Myeloperoxidase.*

Lactoferrin liberation seems also higher in hypoxia than in normoxia in individuals containing the Z allele. Looking into detail in the different phenotypes, we can observe that there amount of lactoferrin in MM is similar in hypoxia and in normoxia, while in all the other phenotypes we can observe a not significant tendency to increase of the protein liberation in hypoxia compared with normoxia. ANOVA test to prove differences among the ratios gave a P-value of 0.9214, meaning that there are no statistical differences among the phenotypes (Figure 15).
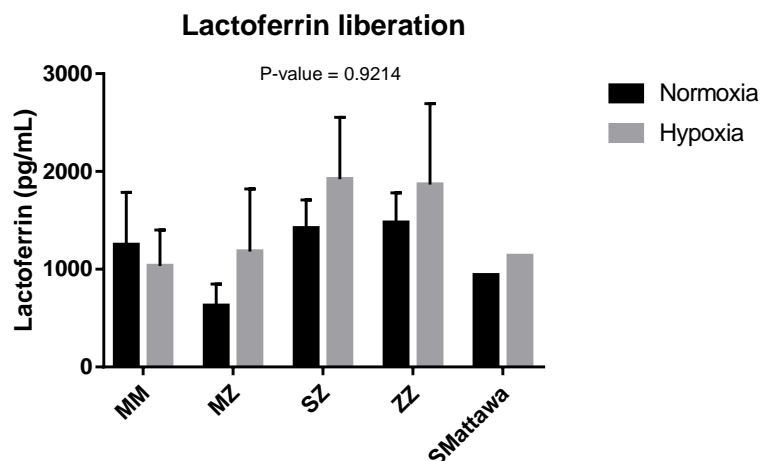

Figure 15. Liberation of lactoferrin to the media in normoxia and hypoxia. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1.

4.5. Oxidative stress markers

Among the measurements in the flow cytometer, two different sets of data can be found, (1) the fluorophores with only basal measurements, and (2) both basal and induced measurements. The differences between normoxia and hypoxia are represented as the mean of the ratio normoxia/hypoxia ± SEM. Values higher than 1 mean more fluorescence in hypoxia than in normoxia and vice-versa. Data with induced measurements are also presented as the mean of the ratio normoxia/hypoxia ± SEM. In this case, FU values of normoxia and hypoxia were transformed into relative fluorescence units, which means that basal measurement is divided by the induced measurement. Induced measurement is taken as the "maximum fluorescent value" obtainable. When presenting the differences, P-value [Phenotype] is the P-value of the ratio from the named phenotype compared to the control individuals (MM) and P-value [ANOVA] is the P-value of the ANOVA test among the phenotypes.

When comparing the oxidative markers, non-significant differences are shown among thiol measurements (P-value ANOVA = 0.9869) (Figure 16.A.). GSH ratio is similar to 1 in all the phenotypes, meaning that the differences are minute in hypoxia and normoxia. MM individuals show the highest mean compared to other phenotypes (P-value ANOVA = P=0.3454) (Figure 16.C.).
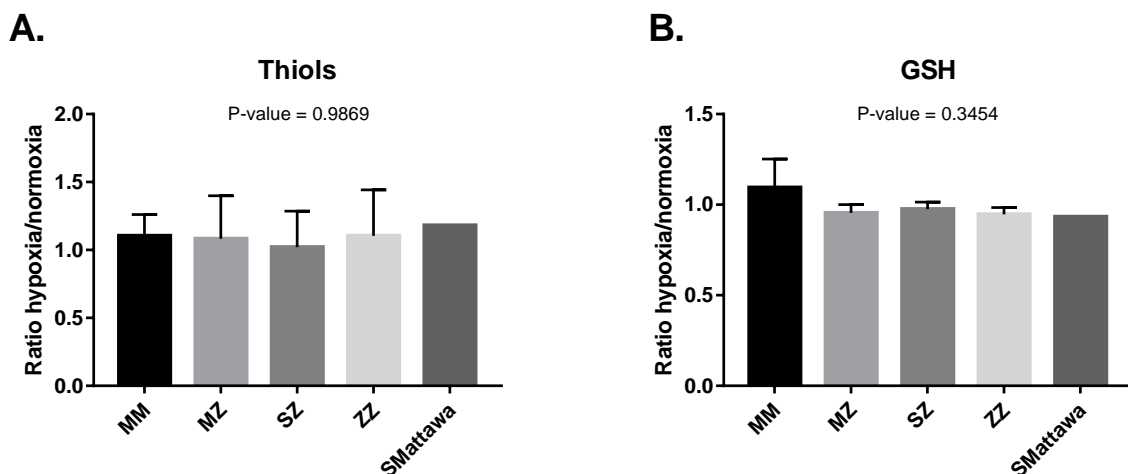
A.

B.

Figure 16. Measurements of (A) Thiols, (B) GSH. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. **GSH** *= reduced glutathione. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1.*

When it comes to H_2O_2, MM individuals show a higher expression in hypoxia than in normoxia, tendency shared with SZ, SMattawa, and MZ patients, even though in MZ patients, the ratio is significantly higher (P-value MZ = 0.0247). ZZ patients show the opposite tendency, but not significant (Figure 17.A.). O_2^- MM individuals present an increase in hypoxia compared to the rest of phenotypes, and in ZZ it can be observed an increase in hypoxia compared to normoxia, but not significant (P-value ZZ = 0.1607) (Figure 17.B.).
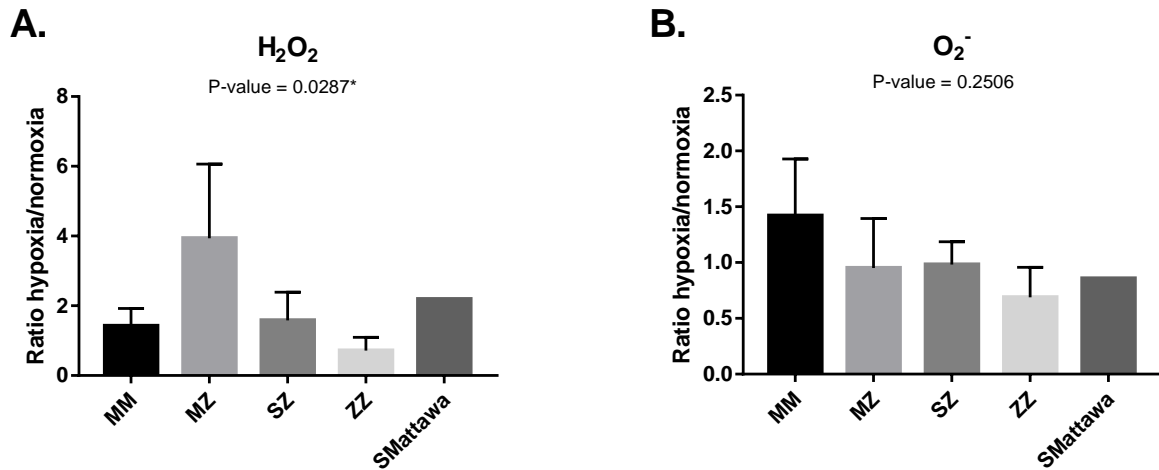
*Figure 17. Measurements of (A) H₂O₂, (B) O₂⁻. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1. * indicates statistical difference.*

As represented in Figure 18.A. MM controls seem to produce a higher amount of NO in hypoxia than the other phenotypes. The highest difference is between MM and MZ (P-value 0.0735). Among the other phenotypes, the difference is lower (P-value ZZ = 0.1536, P-value SZ = 0.1211). ONOO⁻ are in a higher concentration in normoxia than in hypoxia in MM, ZZ and SMattawa individuals; MZ and SZ are at a higher concentration in hypoxia, but there are no significant differences among the ratios (P-value ANOVA = 0.8292) (Figure 18.B.).
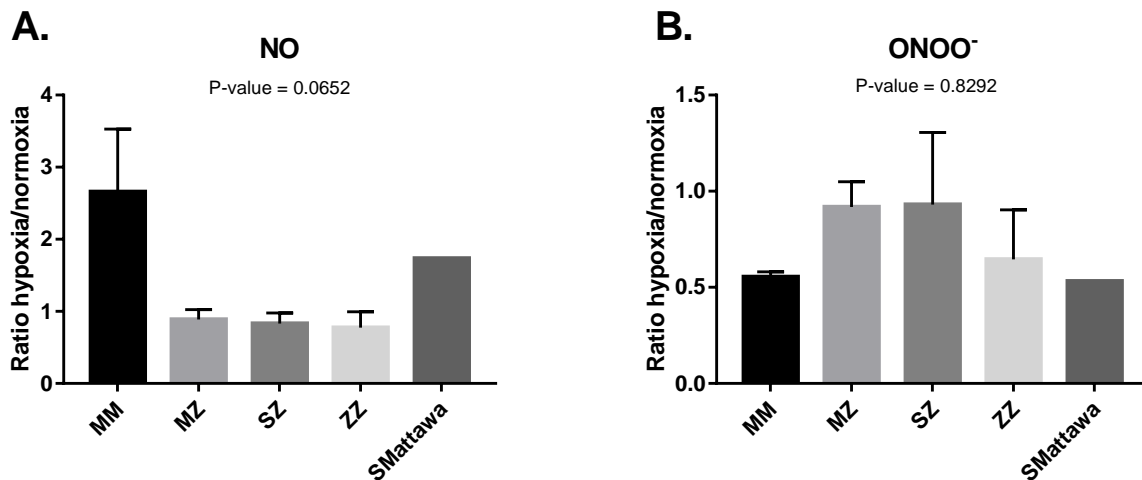

*Figure 18. Measurements of (A) NO, (B) ONOO⁻. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. **NO** = nitric oxide; **ONOO⁻** = peroxynitrites. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1.*

Mitochondrial O_2^- show a tendency to increase are minute among the phenotypes and between normoxia and hypoxia, so it is not possible to observe any tendencies from them (P-value ANOVA = 0.2370) (Figure 19.A.). $\Delta\psi_m$ ratio seems to be higher in MM phenotypes compared to the others. It is significantly lower in ZZ (P-value ZZ = 0.0109) and SZ (P-value = 0.0014) (Figure 19.B.). Intracellular Ca^{2+} is expressed equally in hypoxia and normoxia in MM, MZ, and ZZ. SZ, and SMattawa show a higher concentration in hypoxia than in normoxia (P-value SZ = 0.2118) (Figure 19.C.).
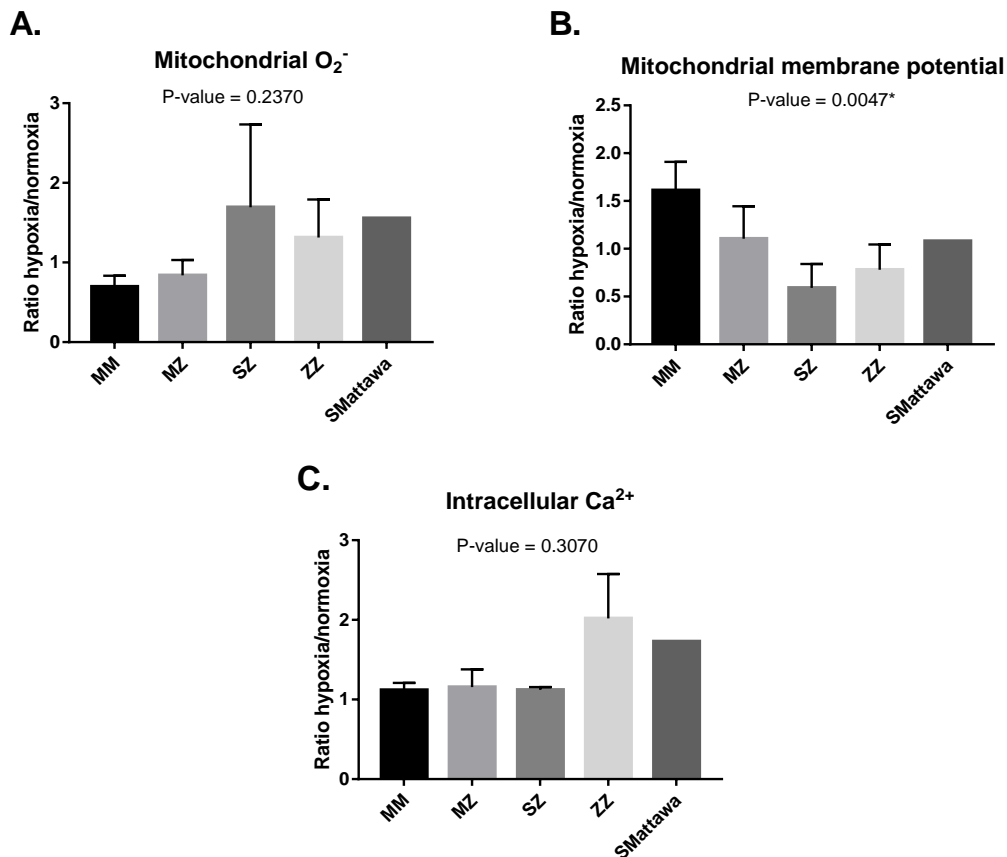
Figure 19. Measurements of (A) mitochondrial O_2^-, (B) mitochondrial membrane potential (C), intracellular Ca^{2+}. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1. * indicates statistical difference.

Looking into oxidative damage markers, carbonylated proteins show some not significant differences (P-value ANOVA = 0.5192). Carbonylation ratio is higher in hypoxia in MM, SZ, and SMattawa, than in hypoxia in MZ and ZZ individuals (Figure 20.A.). Lipids oxidation is higher in normoxia MM than in the other phenotypes analyzed, but differences were not significant neither (P-value ANOVA 0.1838). The widest difference was detected in MZ individuals (P-value MZ = 0.1947) (Figure 20.B.).
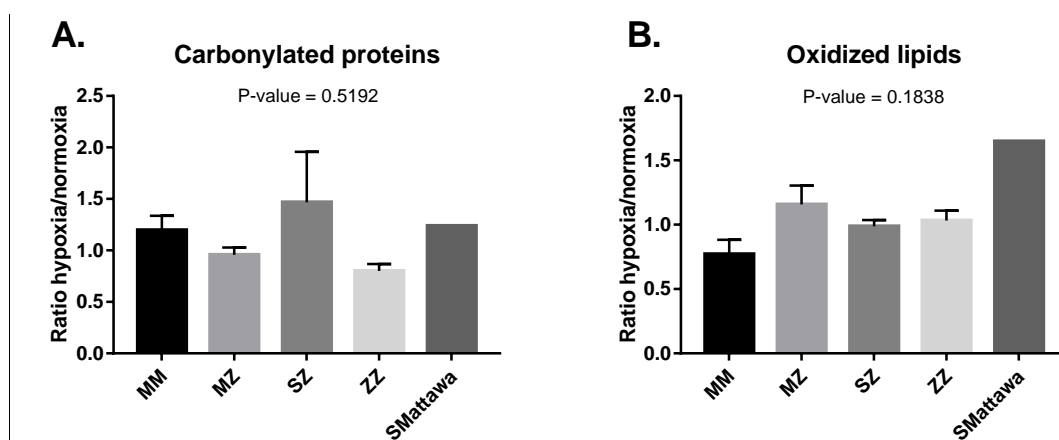

Figure 20. Measurements of oxidative damage in (A) carbonylated proteins, (B) Oxidized lipids. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. n MM = 5, n MZ = 7, n ZZ = 3, n SZ = 5, n SMattawa = 1.

4.6. RT-qPCR Results

Gene fold change were obtained comparing the Cts to the housekeeping gene GADPH (Figure 21). Regarding the genes that regulate hypoxia response, it can be observed that ARNT1 gene tends to be more expressed in hypoxia than in normoxia, but no significant differences among groups are shown (Figure 21.A.). ARNTL does not show differences in hypoxia and normoxia nor the different phenotypes (Figure 21.B.). HIF1A expression is similar between hypoxia and normoxia but seems to be augmented in the Z phenotypes, especially in hypoxia (Figure 21.C.). NFE2L2 shows similar values in all the phenotypes in hypoxia and normoxia except in MZ, where it is less expressed in hypoxia (Figure 21.D.).

Figure 21. Fold change mean expression using ΔΔCt method with GADPH as housekeeping gene. Genes studied were (A) ARNT1, (B) ARNT, (C) HIF1A, (D) NFE2L2. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype.

Gene fold of GCLC values is very low in MM, where it should present values close to 1, but its value is higher in MZ, MZ, SZ, and SMattawa in a not significant manner (Figure 22.A.). GCLM is higher in normoxia than in hypoxia in MM, MZ, and SMattawa; but MZ and ZZ show the highest values are reached in hypoxia. It is significantly less expressed in ZZ (Figure 22.B.). GPX1 shows a lower expression in hypoxia than in normoxia consistently in all the phenotypes (Figure 22.C.). GSR is lower in hypoxia than in normoxia in MM, whereas in MZ, SZ, ZZ and SMattawa show the opposite tendency (Figure 22.D.).
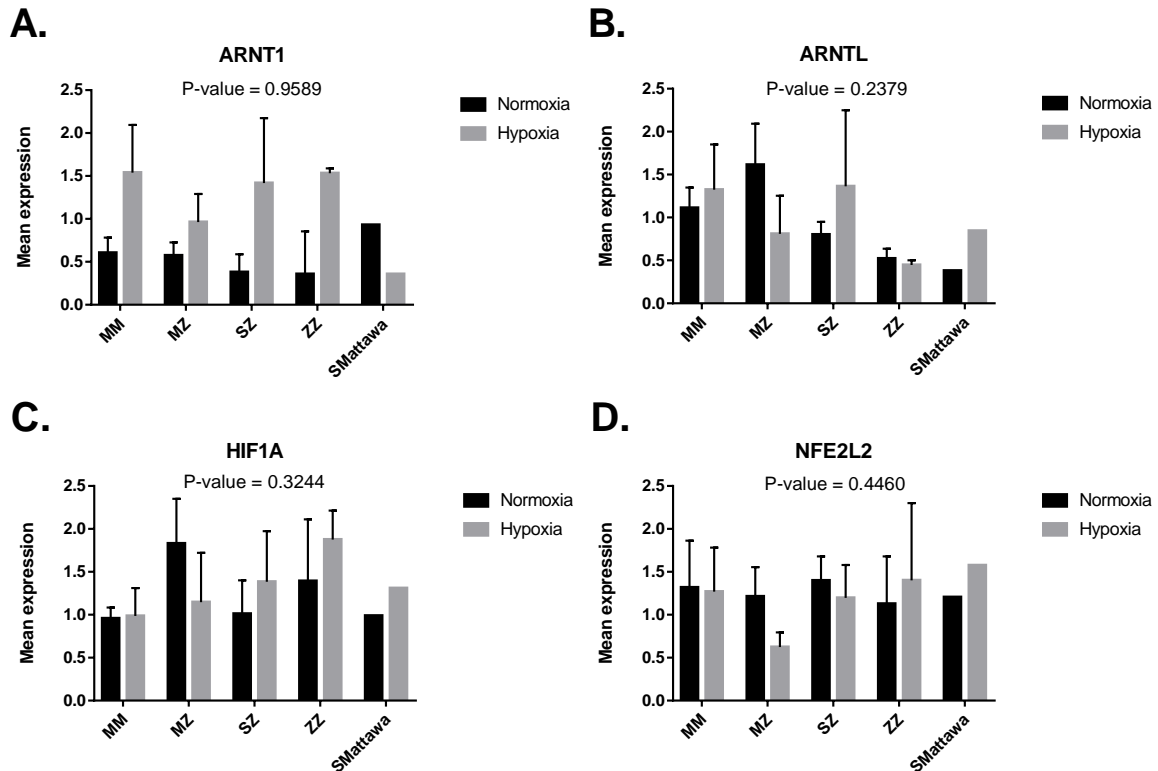
Figure 22. Fold change mean expression using ΔΔCt method with GADPH as housekeeping gene. Genes studied were (A) GCLC, (B) GCLM, (C) GPX, (D) GSR. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype.

SOD1 is expressed at the same levels in hypoxia and in normoxia in all the phenotypes, but in SMattawa shows a much higher gene fold value (Figure 23.A.). SOD2 is highly inconsistent in its values. It can be spotted that in MM controls is expressed the same in hypoxia than in normoxia and in the other phenotypes. Nevertheless, it can be perceived that it changes from one to another phenotype (Figure 23.B.). CAT is similar to MM in all the phenotypes except in ZZ, where it seems to be augmented (Figure 23.C.).
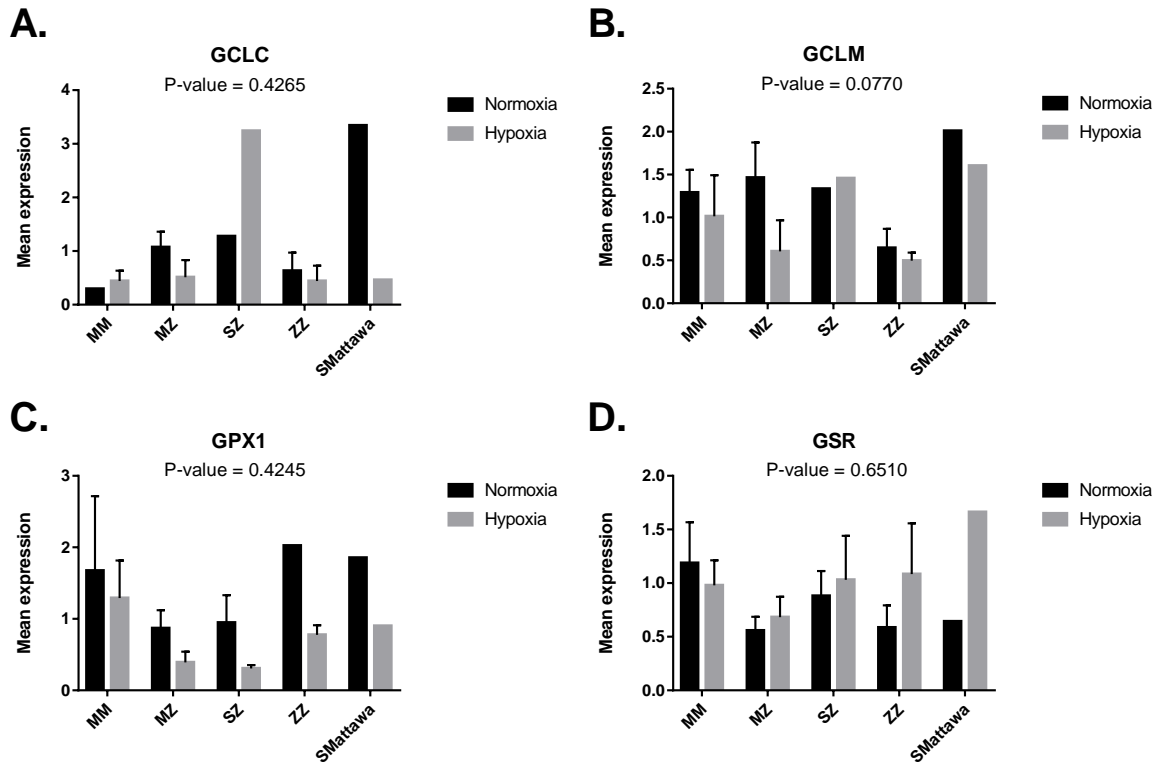
C.

CAT

P-value = 0.0006*

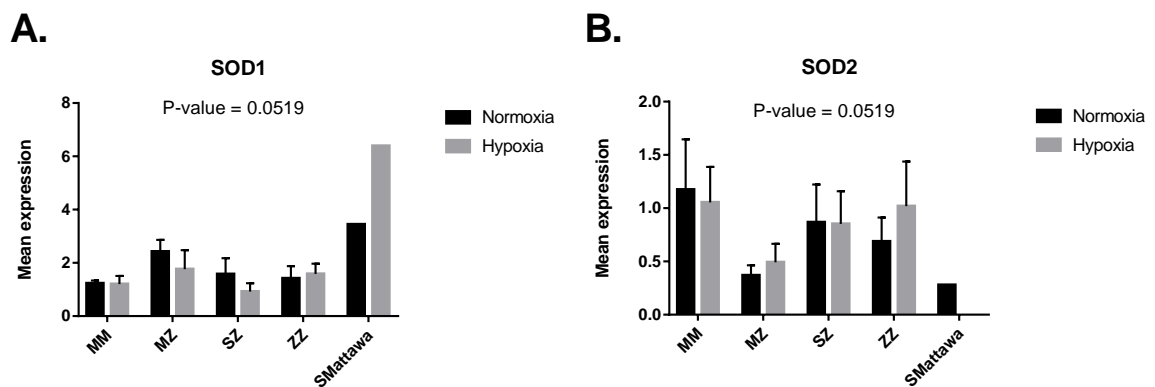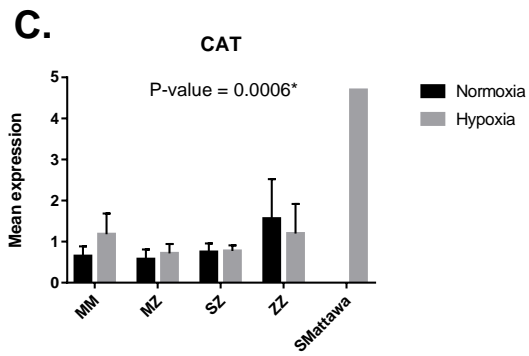*Figure 23. Fold change mean expression using ΔΔCt method with GADPH as housekeeping gene. Genes studied were (A) SOD1, (B) SOD2, (C) CAT. MM stands for controls, SZ for low-risk phenotype and ZZ for high-risk phenotype. MZ have only one deficient allele and SMattawa is a high-risk phenotype. * indicates statistical difference.*

5. Discussion

As this protocol was set up entirely during this final degree thesis, several trials needed to be performed, resulting in successive changes in the different steps. Revisions to the method were achieved using volunteers' blood and undergoing the procedure initially thought. However, its inclusion as MM patients was discarded because of slight differences to the final protocol.

The controls for this study far exceed the mean age of the other phenotypes due to the difficulties explained before. However, we will address this problem including more controls in the near future from different hospitals and by contacting the Spanish registry of patients with alpha 1 antitrypsin deficit (REDAAT). Clinical data shows normal values for hepatic and pulmonary tests, which is expectable in children, even though two ZZ individuals underwent hepatopathy, but they were successfully resolved during the infancy, years before the blood sample was taken.

Regarding the cell type of study, neutrophils are a very delicate cell type that require very strict conditions for avoiding their stimulation. For their isolation, several methods were considered (K Panda and Balachandran, 2013; Oh et al., 2008), but it was decided to perform a negative selection using antibody-coated magnetic beads as it was the method that less affected the activation by manipulation. As a concomitant, this method reduced significantly the time of separation, which provided more time for the subsequent analysis before neutrophils started the apoptosis process. When added to the plate, it was first observed that cells tended to attach to the edges of the wells in the 12-well plate, which could reduce the number of neutrophils in the sample and/or trigger their activation (Lacy, 2006). Sigmacote® allowed the creation of a hydrophobic layer, avoiding the attachment and increasing the final number of neutrophils extracted.

This cell type is so sensitive to stimuli that results obtained with the Lowry assay, confirm the liberation of proteins is higher after the culture in hypoxia (See Figure 12), fact that has been previously reported (Hoenderdos et al., 2016). However, some differences have to be pointed out; three different culture stimulation at time 4h are performed in their essays: with granulocyte and macrophages colony stimulated factors (GM-CSF) 10 ng/mL, platelet activating factor (PAF) 1 µM or TNFα at 20 ng/mL. Among them, TNFα is especially interesting as it has been demonstrated that AAT modulates its activity (and other pro-inflammatory molecules as IL-1) by reducing its secretion and intracellular concentration through the inhibition of the calpain activity (Lockett et al., 2013). Those mechanisms are not present in low circulating AAT concentration, leaving AATD patients unprotected against pulmonary inflammation and avoiding its resolution. Accordingly, and even TNFα showed the most variable results for culture stimulation (Hoenderdos et al., 2016), it was the selected stimulator for the neutrophils culture. Furthermore, the presence of TNFα is exacerbated in the surroundings of the pulmonary tissue in patients with AATD (Bergin et al., 2014) legitimating its use for a more precise *in vitro* model of the neutrophils conditions in patients.

As it was mentioned before, the usage of TNFα as a degranulation inductor provides an uncertain stimulation, already described by Hoenderdos and colleagues (Hoenderdos et al., 2016). Even though some tendencies can be observed, more patients are needed in order to obtain more robust data. In terms of elastase liberation, in hypoxic conditions ZZ and SZ patients nearly double the amount of elastase liberated by MM and MZ. This is very interesting,

as their degranulation seems to be augmented. This has not been reported in the literature on pediatric patients and can be a starting point for future research. (Figure 13) Another degranulation marker, MPO, shows different tendencies in homozygous and heterozygous patients, but it is generally augmented compared to the controls. Although the results did not reach statistical significance. The inclusion of a higher number of patients should confirm the results. Interestingly, SMattawa patient nearly lacks MPO liberation. As there are no studies regarding this result, more experiments including a higher number of patients will need to be carried on (Figure 14). Lactoferrin shows an increase in individuals containing Z allele, however, more experiments will need to be carried on as the low number of patients is a big handicap due to the high variability of the results (Figure 15). In summary, hypoxia induces neutrophil degranulation in AATD patients, although a higher number of patients is needed to obtain statistically significant results in the degranulation markers.

Flow cytometry experiments vary depending on the quantity and type of cells. During the development of a new protocol, several factors need to be taken into account, as the type of culture, concentration, and purity. The method followed for measuring oxidative markers derives from a whole blood cytometry analysis, where many more populations and factors interact with the fluorophores used. Specific antibodies are then mandatory for posterior classification of populations, nevertheless as the pure neutrophil culture means a much more simplified media, the minute quantity of non-neutrophilic particles detected made the usage of the antibodies to be considered negligible, so further experiments were carried separating the cells only by size and morphology, leaving the antibodies for other procedures.

Among the fluorophores, CMFDA was initially used for measurement of GSH present, but it was ulterior substituted by MCB. CMFDA has been reported to show the total levels of free intracellular thiol in other cell lines as neuroblastoma and neurons. Furthermore, it was found that intracellular unbound dye affected the fluorescent background in mouse carcinoma cell lines, which could affect also some other cell types (Sebastià et al., 2003). Nevertheless, both of the fluorophores show equivalent results, which allows us to infer a close relation between total reduced thiols and GSH in neutrophils (Figure 16).

H_2O_2 and O_2^- (Figure 17) are by themselves very powerful oxidants and central precursors to extremely potent oxidants. All the phenotypes but MZs showed a very similar ratio hypoxia/normoxia within both oxidants. MM seem to have it increased in hypoxia, meanwhile, ZZ act as the opposite. An unusual feature is that cytosolic O_2^- and mitochondrial O_2^- seem to behave in a conflicting way (Figure 19). This will need further research in order to investigate a possible causality, as literature theorizes the opposite (Indo et al., 2015).

Another interesting relation is between mitochondrial O_2^- and $\Delta\psi_m$ (Figure 19). The more increased mitochondrial O_2^- is, the fewer $\Delta\psi_m$ can be observed. We interpret the elevated concentration of mitochondrial superoxide anion as an indication of oxidative stress. As O_2^- is swiftly converted into H_2O_2, it escapes from the mitochondria, and damages the organelle in the process, reducing the $\Delta\psi_m$. Nevertheless, as the redox capacity is larger in cytosol than in the mitochondria, antioxidants can eliminate the ROS generated successfully, otherwise, H_2O_2 would be highly accumulated in the cytosol (Maharjan et al., 2014).

Among the gene expression results, we must highlight ARNT1 and which is higher expressed in hypoxia in the controls than in the phenotypes (Figure 21). ARNT1 and ARNTL codify for the hypoxia-inducible factor subunit beta, which dimerizes with the alpha subunit in order to accumulate in the nucleus and activate its target genes, related to hypoxia responses. In

neutrophils, zebrafish models proved that HIF 2α is related to an increase of the lifespan of the neutrophil by avoiding apoptosis (Thompson et al., 2014), so activation of this surviving pathway is alarming for AATD patients, as their defenses against neutrophil-liberated proteases are crippled. HIF1A also shows a slight increase in hypoxia, which is concordant with its function; activate hypoxia-response genes (Figure 21). The expression of the antioxidant enzymes does not show any significant variation among hypoxia or normoxia except the tendency in GPX (Figure 22), which is less expressed in hypoxia in all the phenotypes. In addition, when the phenotypes are compared with MM, they also show a low expression, but no information regarding this tendency has been found in literature.

6. Limitations

The low number of patients and controls analyzed does not allow the data to be robust enough for obtaining conclusive results in gene expression and cytometry. More patients are required and will be obtained from the following hospitals:

- Hospital de Manises (Valencia, Spain)
- Hospital General de Valencia (Valencia, Spain)
- Hospital de Vigo (Pontevedra, Spain)
- Hospital de Granada (Granada, Spain)

The hospitals on the list also participate in the current project and can inform the patients about this project. In addition, the REDAAT can be contacted for recruiting more volunteers.

One of the biggest limitations was obtaining healthy controls suitable for the experiment on behalf of the unlikely scenario it is to extract blood in a pediatric medical revision, since healthy people do not go to hospitals.

7. Conclusions

- During the realization of this final master's degree project, a new strategy for the study of the activation, degranulation and oxidative profile has been established for the realization of the project. As the protocols are set up, more volunteers would be able to join the study and add robustness to the tendencies here observed.
- A significant increase in protein liberation was observed after culturing neutrophils 4h in hypoxia. Among the degranulation markers it is possible to observe:
  - Elastase liberation was nearly doubled in SZ and ZZ patients compared to MM controls.
  - MPO was also augmented in patients except in SMattawa, where its liberation is minute.
  - Lactoferrin seemed to be augmented in patients containing the Z allele compared to the MM controls.
- An interesting relationship between increased mitochondrial O_2^- and lowered $\Delta\psi_m$ has been detected and will be studied more in depth, as varies among the phenotypes.
- ARNT1 gene is consistently more expressed in hypoxia than in normoxia, accordingly to its function. HIF1A shows an increasing tendency in patients containing the Z allele in hypoxia compared to the controls.

- AATD shows a highly variable clinical phenotype and the existence of modifiers that may influence disease progression has been proposed. According to this and previous work conducted by the group, results suggest that it might be worth studying oxidative stress, oxidative defense response and telomere length regulation as potential genetic factors relevant to the severity of the disease.

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9. Annexes

Annex 1 Patient information sheet and informed consent.

GENERALITAT VALENCIANA
CONSELLERIA DE SANIDAD UNIVERSAL Y SALUD PÚBLICA

Hospital Clínic Universitari

DEPARTAMENT CLÍNIC MALVA-ROSA

HOJA DE INFORMACIÓN AL PACIENTE PEDIÁTRICO (Menores de 12 años)

Se te ofrece la posibilidad de participar en el proyecto de investigación titulado "**Estudio del efecto de la hipoxia en la degranulación, en la producción de citoquinas y en el perfil oxidativo de los neutrófilos de pacientes con déficit de alfa-1 antitripsina**" que está siendo realizado por los **Drs. Amparo Escribano Montaner** y **Francisco Dasí Fernández** del Servicio de **Pediatría** y que ha sido ya evaluado y aprobado por el Comité Ético de Investigación Clínica del Hospital Clínico Universitario de Valencia.

Antecedentes
El déficit de alfa-1-antitripsina (DAAT) es un trastorno metabólico de carácter hereditario (que se transmite de padres a hijos) que está causado por cambios en una proteína que se llama alfa-1 antitripsina (AAT), la cual se produce principalmente en el hígado. La principal función de la AAT es proteger al tejido pulmonar del daño causado por una proteína llamada elastasa. A causa de esto, los pacientes pueden llegar a desarrollar problemas pulmonares y en el hígado que en algunos casos pueden ser graves. No obstante, debido entre otras causas al bajo número de pacientes afectados, no se dispone de tratamientos eficientes y definitivos que consigan mejorar la calidad de vida de los pacientes que la padecen. Investigaciones recientes han descubierto que los neutrófilos (un tipo de célula de la sangre) puede desempeñar un papel importante en el daño pulmonar que se observa en algunos pacientes. Estas células cuando se activan en condiciones de falta de oxígeno (hipoxia) contribuyen al daño pulmonar.

¿Cuál es el objetivo de este estudio?
El objetivo del presente proyecto es estudiar el efecto que tiene la falta de oxígeno en la evolución de la enfermedad y evaluar el efecto de los tratamientos de los que actualmente disponemos en la activación de los neutrófilos.

¿Por qué se le ha pedido que participe?

Se te pide que participes en este estudio ya que tienes **Déficit de alfa-1 antitripsina**.

¿En qué consiste tu participación? ¿Qué tipo de pruebas o procedimientos se te realizarán?

Se te solicita permiso para utilizar con fines científicos **una muestra de sangre**.
La participación en el presente proyecto no supone ninguna alteración del tratamiento que estés llevando (si lo tiene) y todo tratamiento que se te pueda poner será siempre bajo criterio médico.

¿Cuáles son los riesgos generales de participar en este estudio?

El único riesgo previsible de tu participación será el mínimo riesgo que conlleva la extracción de una muestra de sangre, que incluye molestias, dolor, enrojecimiento e hinchazón y/o pequeños hematomas en el lugar del brazo donde se ha producido la extracción.

¿Cuáles son los beneficios de la participación en este estudio?

Es muy posible que los resultados obtenidos en esta investigación tengan poco valor diagnóstico o predictivo para ti, pero podrá ayudar a conocer mejor su enfermedad y mejorar el pronóstico y el tratamiento de futuros pacientes.

¿Qué pasará si decido no participar en este estudio?

Tu participación en este estudio es totalmente voluntaria. En caso de que decidas no participar en el estudio, esto no modificará el trato y seguimiento que de tu enfermedad realicen ni su médico ni el resto del personal sanitario que se ocupa de su enfermedad. Asimismo, podrás retirarse del estudio en cualquier momento, sin tener que dar explicaciones.

¿A quién puedo preguntar en caso de duda?

Es importante que comentes con cualquiera de los investigadores de este proyecto las dudas que surjan antes de firmar el consentimiento para tu participación. Asimismo, podrás solicitar cualquier explicación que desees sobre cualquier aspecto del estudio y tus implicaciones a lo largo del mismo, contactando con los investigadores que están realizando el proyecto, los **Drs. Amparo Escribano Montaner** en el teléfono **961 973 879** o el **Dr. Francisco Dasi Fernández** en el teléfono **676 515 598**.

Confidencialidad:

Todos sus datos, así como toda la información médica relacionada con tu enfermedad será tratada con absoluta confidencialidad por parte del personal encargado de la investigación. Asimismo, si los resultados del estudio fueran susceptibles de publicación en revistas científicas, en ningún momento se proporcionaran datos personales de los pacientes que han colaborado en esta investigación.
Tal y como contempla la Ley Orgánica 15/1999 de Protección de Datos de carácter personal, podrás ejercer tu derecho a acceder, rectificar o cancelar sus datos contactando con el investigador principal de este estudio.

¿Qué pasará con las muestras biológicas obtenidas durante la investigación?

Durante tu participación en este estudio, se te extraerá una muestra de sangre periférica.

Esta muestra será siempre utilizada con fines científicos, pudiéndose utilizar si lo autoriza en el marco de otros proyectos de investigación que tengan como objetivo el estudio de su enfermedad y que previamente hayan sido evaluados y aprobados por el Comité Ético de Investigación Clínica del Hospital.
Dicha muestra será conservada en una colección registrada en el ISCIII ubicada en la Unidad Central de Investigación de la Facultad de Medicina piso 2E en un congelador de -80 °C durante un periodo de tiempo de 4 años.

Además, este material no será bajo ningún concepto ni en ningún momento motivo de lucro, bien sea por la venta del material o de los derechos para realizar estudios sobre los mismos.

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DEPARTAMENT CLÍNIC MALVA-ROSA

CONSENTIMIENTO INFORMADO

Título del Proyecto titulado: **Estudio del efecto de la hipoxia en la degranulación, en la producción de citoquinas y en el perfil oxidativo de los neutrófilos de pacientes con déficit de alfa-1 antitripsina.**

Investigador principal: **Dra. Amparo Escribano Montaner**

Servicio: Pediatría

Yo, _____ he sido informado por el Dr._____, colaborador del proyecto de investigación arriba mencionado, y declaro que:

- He leído la Hoja de Información que se me ha entregado
- He podido hacer preguntas sobre el estudio
- He recibido respuestas satisfactorias a mis preguntas
- He recibido suficiente información sobre el estudio

Comprendo que mi participación es voluntaria

Comprendo que todos mis datos serán tratados confidencialmente

Comprendo que puedo retirarme del estudio:
- Cuando quiera
- Sin tener que dar explicaciones
- Sin que esto repercuta en mis cuidados médicos

SE RECUERDA QUE EL APARTADO SIGUIENTE ÚNICAMENTE SE DEBE MANTENER CUANDO PROCEDA.

Autorizo a que las muestras obtenidas durante el proyecto de investigación sean utilizadas con fines científicos en otros proyectos de investigación que tengan por objeto el estudio de mi enfermedad y que hayan sido aprobados por el Comité de Ética de Investigación Clínica del Hospital Clínico Universitario de Valencia

☐ Sí ☐ No

Quiero que se me pida autorización previa para utilizar mis muestras biológicas para futuros proyectos de investigación

☐ Sí ☐ No

Con esto doy mi conformidad para participar en este estudio,

Firma del paciente: Firma del Investigador:
Fecha: Fecha

Annex 2. Funding