Carlota Moutinho Pascoal

Licenciada em Bioquímica

Immunological aspects of glycosylation: from aberrant to defective glycosylation

Dissertação para obtenção do Grau de Mestre em

Bioquímica para a Saúde

Orientador: Paula Videira, Profesora Auxiliar, FCT/UNL

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Faculdade de Ciência e Tecnologia – Universidade Nova de Lisboa

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Immunological Aspects of Glycosylation: from aberrant to defective glycosylation

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FCT-UNL, UNL

The work developed during this master project has originated:

- **Oral presentations:**

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- **Posters:**

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- **Patient-friendly document (Portuguese and English version, under graphic design with expected publication by the end of 2017):**

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“Don’t limit yourself.

Many people limit themselves to what they can do.

You can go as far as your mind lets you.

What you believe, remember, you can achieve.”

Mary Kay Ash

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Abstract

Glycosylation is crucial in many biological processes, like cell recognition, signaling and development. Many diseases present altered glycosylation and two extremes are cancer and congenital disorders of glycosylation (CDG), with aberrant and defective glycosylation, respectively.

Sialic acids are glycans' terminal sugars with an immunomodulatory role and when decreased, typically activate immune cells, as dendritic cells. Interestingly, both ST6Gal-I and its derived α2,6 sialylation are overexpressed in cancer. Here, we hypothesized that cancer cells secret functional ST6Gal-I that modulates immune cells' glycosylation and their activity as a cancer immune evasion mechanism.

Also interestingly, patients with PMM2-CDG (the most frequent CDG type) present immunological affectation. Here, we hypothesized that the PMM2-CDG-defective glycosylation observed also influences the function of immune cells.

Therefore, the main goals of this study comprised the assessment of the immunological aspects of cancer cells and CDG glycosylation. Specifically, we intended to (1) study the expression and secretion of ST6Gal-I by colorectal cancer (CRC) cells and test its function in modulating immune cells activity; (2) develop a PMM2-CDG leukocyte cell line as a model to unravel patients' immunity and to evaluate their response to mitogenic stimulation. Moreover, as PMM2-CDG have a profound impact in patients' quality of life (QoL), patient and observer reported outcomes measures (PROMs and ObsROMs) were reviewed. These may integrate primary endpoints in clinical trials to find treatment to PMM2-CDG.

Our data demonstrated that (1) CRC cells secret ST6Gal-I enzyme, however further work is needed to evaluate its role in immune modulation; (2) PMM2-CDG T cells have higher proliferation capacity and IFN-γ cytokine expression, in response to a mitogen as compared to the healthy control and (3) there are significant numbers of tools for future evaluation of PMM2-CDG patients' and caregivers' QoL.

This study may contribute to better understand the glycan-related pathological mechanisms.

Keywords:

Immunomodulatory role of glycosylation, ST6Gal-I, extrinsic α-2,6-sialylation, Congenital Disorders of Glycosylation (CDG), PMM2-CDG, patient reported outcomes measures (PROMs).

Resumo

A glicosilação é crucial em muitos processos biológicos, como o reconhecimento, sinalização e desenvolvimento celular. Muitas doenças apresentam glicosilação alterada e dois extremos são o cancro e doenças congénitas da glicosilação (CDG), com glicosilação aberrante e defeituosa, respectivamente.

Ácidos siálicos são açúcares terminais de glicanos com papel imunomodulatório e, quando diminuídos, tipicamente ativam células imunes, como as células dendríticas. Curiosamente, tanto o enzima ST6Gal-I como a sua derivada sialilação α2,6 estão sobreexpressas em cancro. Aqui, pôs-se a hipótese que as células cancerígenas secretam ST6Gal-I que modula a glicosilação das células imunes e a sua atividade como um mecanismo de evasão imunológica.

Por outro lado, pacientes com PMM2-CDG (o tipo de CDG mais frequente) apresentam envolvimento imunológico. Aqui, pôs-se a hipótese que a glicosilação defeituosa observada em PMM2-CDG também influencia a função das células imunes.

Assim, os principais objetivos deste estudo abrangeram a avaliação dos aspectos imunológicos da glicosilação em cancro e CDG. Especificamente, pretendemos (1) estudar a expressão e secreção de ST6Gal-I por células de cancro colorectal (CRC) e testar a sua capacidade de modular a atividade das células imunes; (2) desenvolver uma linha celular leucocitária PMM2-CDG como um modelo para desvendar a imunidade dos pacientes e avaliar a sua resposta a uma estimulação mitogénica. Para além disso, como a PMM2-CDG tem um profundo impacto na qualidade de vida (QoL) dos pacientes, foram revistos *patient* e *observer reported outcomes measures* (PROMs e ObsROMs). Estas medidas podem vir a integrar *endpoints* primários em ensaios clínicos para encontrar tratamento para a PMM2-CDG.

Os nossos dados demostraram que (1) células de CRC secretam o enzima ST6Gal-I, contudo, é necessário trabalho futuro para avaliar o seu papel na modulação imune; (2) Células T PMM2-CDG têm uma capacidade de proliferação e expressão da citocina IFN-γ mais elevadas em resposta a uma estimulação mitogénica em comparação com o controlo saudável e (3) existe um número significante de instrumentos para futura avaliação da QoL de pacientes PMM2-CDG e dos seus cuidadores.

Este estudo pode contribuir para uma melhor compreensão dos mecanismos patológicos relacionados com glicanos.

Palavras-chave:

Papel imunomodulatório da glicosilação, ST6Gal-I, sialilação extrínsica α2,6, Doenças Congénitas da Glicosilação (CDG), PMM2-CDG, *patient reported outcomes measures* (PROMs).

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List of Abbreviations

Ab(s)	Antibody (antibodies)
APC	Antigen presenting cells
Apc	Allophycocyanin
APR	Acute phase response
APP	Acute phase proteins
Asn	Asparagine
ATM	Ataxia telangiectasia mutated Ser/Thr kinase
BACE 1	Beta-site-amyloid precursor protein-cleaving enzyme 1
CD	Cluster of differentiation
CDG	Congenital Disorders of Glycosylation
CFDA-SE	Carboxifluorescein diacetate succinimidil ester
CFSE	Carboxyfluorescein succinimidyl ester
Chk2	Checkpoint kinase 2
CMP-Sia(s)	Cytidine-5'-monophospho-sialic acid(s)
CRC	Colorectal Cancer
C_T	Threshold cylcle
DAG	Diacylglycerol
DAMP(s)	Damage-associated molecular pattern(s)
(mo-)DC(s)	(monocyte-derived) Dendritic cell(s)
DDR(s)	Deoxyribonucleic acid damage response(s)
(c)DMEM	(complete) Dulbecco's Modified Eagle Medium
(c)DNA	(complementary) Deoxyribonucleic acid
EBV	Epstain Barr Virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FITC	Fluorescein isothyocyanate
FBS	Fetal bovine serum
FSC-A	Forward scatter - area
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GalNAc	N-acetylgalactosamine
(Galβ1-4)GlcNAc	(Galactose β1-4) N-acetylglucosamine
HI	High intensity
HLA	Human leukocyte antigen

HRP	Horseradish peroxidase
(HR)QoL	(Health related) Quality of life
HSP(s)	Heat-shock protein(s)
IFN	Interferon
Ig(s)	Immunoglobulin(s)
IL(-XR)	Interleucin(-X receptor)
Ion	Ionomycin
IP_3	Ionositol triphosphate
LCL(s)	Lyphoblastic cell line(s)
LI	Low intensity
MAPK	Mitogen-activated protein kinase
MHC (-I /-II)	Major histocompatibility complex (class I/ class II)
MW	Molecular weight
Neu5Ac	N-acetylneuraminic acid
NFAT	Nuclear factor of activated T cells
NK	Natural killer
ObsROM(s)	Observer reported outcome measure(s)
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood monocuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI(s)	Proliferation index(indexex)
PIP_2	Phosphotidylinositol 4,5 bisphosphate
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMM2	Phosphomannomutase 2
PROM(s)	Patient reported outcome measure(s)
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
RDBMS	Relational database management system
(m)RNA	(messenger) Ribonucleic acid
(c)RPMI	(complete) Roswell Park Memorial Institute medium
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel eletrophoresis
Ser	Serine
SG	ST6Gal-I deficient

Sia(s)	Sialic acid(s)
SNA	*Sambucus nigra*
SQL	Structured Query Language
ST	Sialyltransferase(s)
ST3Gal	β-galactoside α2,3 sialyltransferase
ST6Gal	β-galactoside α2,6 sialyltransferase
ST6GalNac	N-acetyl-galactosamine α2,6 sialyltransferase
ST8Gal	α2,6 sialyltransferase
TBS-T	Tris buffered saline(-tween)
T_C cells	Cytotoxic ($CD8^+$) T cells
TCR	T cell receptor
T_H cells	Helper ($CD4^+$) T cells
TERT	Telomerase reverse transcriptase
Thr	Threonine
TNF	Tumor necrosis factor
US	Unstained
WB	Western Blot
WT	Wild type

1. Introduction

1.1. Immune system

The immune system can be described as a set of molecules, cells and tissues responsible for conferring protection against foreign bodies to the organism. However, the immune system must have the ability to distinguish self- from non-self molecules, in order to act in a protective manner. The next topics will describe the complexity of the immunological system and its function in the protection of the organism.

1.1.1. Innate immune response

There are two types of immune responses that complement each other. Innate immune response is a non specific first line of attack to pathogens, whether microbes or even toxins, constituted by biological barriers (epithelia and antimicrobial agents), phagocytic cells (neutrophils, macrophages), dendritic cells (DCs), natural killer (NK) cells, among others, and also by molecules such as complement system proteins.[1] The cells that are part of this kind of immunity have pattern recognition receptors (PRRs) that not only recognize moieties known as pathogen associated molecular patterns (PAMPs), but also danger-associated molecular patterns (DAMPs) derived from host cells with changes in homeostasis.[2] These receptors have a broad specificity as they are able to recognize groups of pathogens with the same PAMP.[1] After recognition, transcription factors like nuclear factor κB and interferon (IFN) regulatory factors are activated which triggers the expression of chemokines, cytokines, among others.[3] Chemokines and cytokines (or interleucins – ILs) are secreted proteins that not only promote the recruitment and activation of immune cells which are responsible for the destruction and clearance of pathogens, but also may induce the acute phase response (APR).[1,4] APR is a mechanism of the innate immune response with the aim of reestablishing homeostasis after an immune challenge and is characterized by the modulation of hepatic synthesis of a wide group of proteins. Proteins that have their concentration increased during APR are referred to as positive acute phase proteins (APP), in contrast with negative APP that have their concentration diminished.[4] The innate immune response also has a main role in the activation of the adaptive immune response, as described in the next topic.

1.1.2. Adaptive immune response

The adaptive (or acquired) immune system acts latter on in the immune response. In contrast to innate immune responses, adaptive ones are stimulated by an immunogen (substance that is capable of trigger an immune response) being the response magnitude and efficacy increased after each exposure. The key players in the adaptive response are T and B lymphocytes that have specific receptors that distinguish between similar antigens (immunogen or any other substance that may be

specifically bound to the components of the immune system). As a matter of fact, T and B lymphocytes are capable to distinguish between minimal structural differences in different parts of the same antigen, i.e. antigenic determinants or epitopes. Additionaly, lymphocytes confer immunological memory, being the second contact with the same antigen capable of eliciting an effective and stronger response.[5] B cells recognize the antigen in its native conformation and produce specific antibodies (Abs, also known as immunoglobulins – Igs) to recognize them, whereas T cells are only capable to recognize small and processed peptides from a pathogen in the context of major histocompatibility complex (MHC) molecules, also called human leukocyte antigen (HLA).[1] These molecules can be of two classes: MHC class I (MHC-I) or MHC class II (MHC-II). The first class is expressed by all nucleated cells and present small peptides from cytosolic or nuclear origin.[6] The MHC-I-peptide complex is then recognized by cluster of differentiation (CD) 8^+ T cells (also called effector or cytotoxic T cells – T_C cells) that will kill the cell that produced the intracellular antigen.[6] Besides intracellular antigens or microbes, T_C cells are also responsible for the cytotoxic killing of malignant cells.[7] The MHC-II molecule is expressed in the surface of the so-called professional antigen-presenting cells (APC). These cells include DCs, macrophages and B cells, however only DCs are capable to activate naïve T cells and elicit a primary immune response. The small fragments of proteins presented by these molecules arise from extracellular antigens and are recognized by $CD4^+$ T cells (also called helper T cells - T_H cells).[6] Upon recognition, T_H cells produce a wide set of cytokines that regulate the immune response, whereas the secreted cytokines determines the specific T_H subset.[8] The nature of the immune response is defined by two major T_H subsets, namely T_H1 and T_H2 cells. While T_H1 cells and its main effector cytokine IFN-γ are the basis to cell-mediated immunity, i.e, the recruitment and activation of phagocytes to destroy infected cells or phagocytosed microbes after antigen presentation, T_H2 cells, that mainly produce IL-4, support the humoral immune response, i.e, the B cell-derived antibodies-mediated immunity.[8] Another important T_H subset is regulatory helper T cells (T_{reg}), producers of IL-10, that are responsible for regulating immune responses.[9] In order for T lymphocytes to become activated, besides the need to recognize the antigen, they need co-stimulation by molecules expressed in the DCs surface and the action of cytokines.[10] Not only cytokines are responsible for immune cells recruitment, but also play important roles in the proliferation and differentiation of T and B cells, as well as in the T cells effector functions.[1] Besides the action in differentiation of T_H1 cells, IFN-γ is responsible for the regulation of B cell function, such as antibody production, but also for the immune cells recruitment, activation, maturation and differentiation (i.e. macrophages), directing antimicrobial and anti-tumor mechanisms and enhancing antigen processing and presentation.[11] IL-4 mediates the proliferation of T cells, the alternative activation of macrophages inhibiting the classical one and participates in B cell isotype switching, in addiction to the T_H2 differenciation.[1] IL-10 is an anti-inflammatory cytokine involved in the inhibition of T_H1 cells, NK cells and macrophages to prevent exaggerated inflammatory responses.[12] Although controversial, IL-10 has been proposed to contribute to tumor immune escape (further depicted in point 1.3.3.) due to its immunosuppressive features.[13] As for tumor necrosis factor(TNF)-α, this

cytokine is mainly produced by activated macrophages and lymphocytes in response to inflammatory or infectious conditions. When released, it has a chemotactic effect for the recruitment of leukocytes and promote inflammation features as the expression of endothelial adhesion molecules for leukocyte extravasation to the sites of infection, vasodilatation and expression of pro-coagulant factors.[14]

1.1.3. Mitogen Stimulation

A mitogen is a polyclonal activator substance that triggers signal transduction pathways in which mitogen-activated protein kinase (MAPK) is involved, leading to cell division (mitosis) and proliferation.[15] In contrast with antigens, being polyclonal activators, mitogens activate lymphocytes independently of their antigenic specificity.[16] Therefore, they are often used to stimulate lymphocytes and thereby assess immune function.[17,18]

Activation of human T cells via surface receptors is associated with hydrolysis of membrane phosphotidylinositol 4,5 bisphosphate (PIP_2), resulting in the generation of the second messengers ionositol triphosphate (IP_3) and diacylglycerol (DAG). Subsequently, IP_3 causes the release of Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC) and MAPK pathways. The increase in Ca^{2+} concentration is necessary for the nuclear factor of activated T cells (NFAT) signaling that controls the induction of several cytokine gene expression.[19] Phorbol myristate acetate (PMA) and calcium ionophores, such as ionomycin (Ion), are mitogens widely used to assess immune function.[20,21] PMA is a mitogen which diffuses through the cell membrane into the cytoplasm, where it directly activates PKC mimicking the action of surface receptor-derived DAG.[22,23] Besides activating NFAT signaling, Ion triggers the hydrolisis of phosphoinositides and induces the activation of PKC.[24,25] Several studies highlight the synergistic role of Ca^{2+} with PMA derived from the enhancement of PKC activation.[21,24,26]

1.2. Glycosylation

Glycosylation is the principal eukaryotic post translational modification of proteins and lipids and relies on glycosyltransferases that assemble monosaccharides derived from the primary metabolism into linear or branched glycan chains.[27] Regarding protein glycosylation, there are two main types of protein glycosylation, N- and O-glycosylation.[27] While N-glycosylation gives rise to glycans linked to the amide groups of asparagine (Asn) side chains of a protein, O-glycosylation generates O-glycans which are linked to the oxygen atom of threonine (Thr) or serine (Ser) side chains (Figure 1.1). Both processes are further explained below.

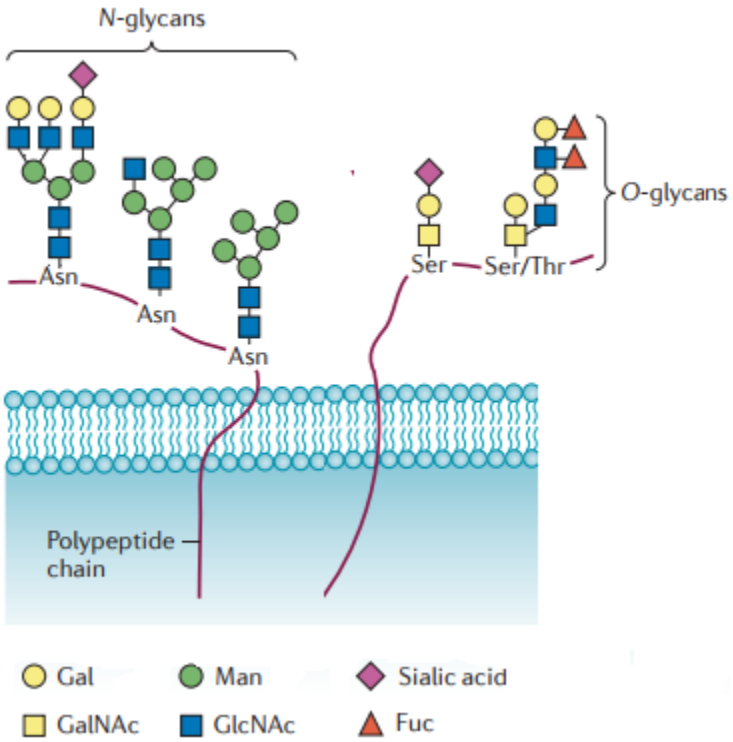

Figure 1.1 – N-glycans and O-glycans. N-glycans are linked via a N-linkage to Asn whereas O-glycans are linked via a O-linkage to Ser/Thr. Adapted from Pinho and Reis 2015.[28]

Glycoconjugates have great diversity depending on the sugars that constitute them and the glycosidic bond involved and have critical biological functions that include protein structural stability, cellular signalling and cell-cell interactions, for instance leukocyte homing.[29–31] N-glycosylation takes place in a two-phase process. The first phase happens in the endoplasmic reticulum (ER) where a precursor oligosaccharide is assembled on a lipid carrier, dolichol pyrophosphate, by the addition of three different monosaccharides, N-acetylglucosamine (GlcNAc), mannose and glucose. After the biosynthesis of this precursor, it is transferred to the nascent protein by the oligosaccharyltransferase via a N-glycosidic linkage of a GlcNac to an Asn residue. However, the Asn residue must be part of the consensus aminoacid sequence Asn-X-Ser/Thr (where X stands for any aminoacid, except proline).[32,33] The second phase of N-glycosylation includes the removal of certain monosaccharides from the protein-attached glycan ("trimming") and the addition of others in the ER or in the Golgi compartment ("processing").[34] Even though there is a pre-defined order in the N-glycoprotein biosynthetic pathway, N-glycan structures diversity is ensured by different expression and organization patterns between cells as well as substrate competition among the enzymes enrolled in this process.[35] O-glycosylation is initiated on the rough ER or the cis Golgi and has a great variability according to the sugars and bond-type involved. It begins with the transfer of N-acetylgalactosamine (GalNAc) from uridine 5'-diphospho-GalNAc to the hydroxyl group of a Ser or Thr residue in the

protein. This Ser/Thr-GalNac structure forms the core to longer and more complex structures. In contrast to N-glycans, O-glycans are small and non-processed structures, being the glycans constructed by single sugar transfers catalyzed by glycosyltransferase enzymes, and not by the transfer of any pre-assembled precursors[36]. The importance of glycosylation in biological systems is well ilustrated by the existance of several diseases that have been associated with glycosylation defects, for instance, cancer, congenital disorders of glycosylation (CDG), Alzheimer's disease as well as Parkinson's disease.[28,37–39]

1.2.1. Glycosylation and its immunological function

It is well known that glycans play an active role in almost every aspect of immunology. In fact, surface immune receptors are glycoproteins and their glycan content dictates their biological function. For instance, T cell receptor (TCR) glycosylation has been shown to modulate functional avidity, receptor clustering and association with other cell surface proteins.[40,41] Glycan-binding proteins constitute another major component of the immune system and are called lectins that include C-type lectins, Siglecs and galectins.[42] The binding of glycans to lectins and Siglecs and the subsequent steric modulation of molecular interactions is involved in either innate and adaptive immune responses.[43] Also, Abs and cytokines are secreted glycoproteins and glycan content can modulate their activity.[44,45] Furthermore, lymphocyte development rely on glycan changes to give rise to the different subsets from the cell progenitors and leukocyte homing upon the recognition of glycans expressed in epithelial sites near infection or inflammation by selectins.[46,47] Moreover, concerning pathogens, the majority of immunogens are glycans or glycoconjugates, for instance bacterial lipopolysaccharides, once more revealing the importance of carbohydrates in immunological function.[42]

1.2.2. Sialic Acids roles in the immune system

Sialic acids (Sias) are a family of nine carbon backbone monosaccharides, usually found in the terminal positions of glycans, which contributes to the huge variety of glycan structures in the cell surface.[48] The most abundant Sia found in humans and also precursor from a great variety of Sias structures is the N-acetylneuraminic acid (Neu5Ac) which bears an acetyl group on the carbon 5 (C5) (Figure 1.2). Chemical diversity of Sias arises from substitution in the C4, C5, C7, C8 and C9 positions and they are bound by different types of α linkages between the C2 of the Sia structure and the terminal sugar of the glycan.[48,49] These different linkages are formed by specific sialyltransferases (STs) (further described in the next topic) that use cytidine-5'-monophospho-Sia (CMP-Sia) as a donor.[48]

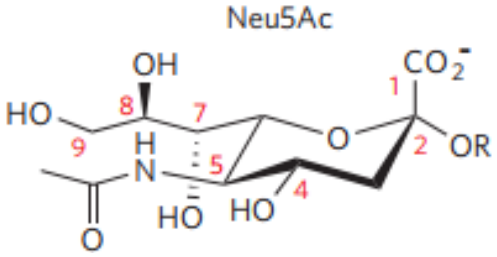

Figure 1.2 - Neu5Ac chemical structure. Adapted from Varki and Schauer 2009.[49]

Sias play an extensive list of biological roles. Their presence in secreted glycoproteins suggests that they have an important action in their stabilization, as it is the case for the secreted adiponectin from adipocytes.[50] Sias electronegative charge influences the binding and transport across membranes due to attractive and repulsive forces, for instance, they can repel proteases and glycosidases preventing degradation of surface glycoproteins.[51] In the immunological field, Sias have a dualistic role. They can act as masks of antigens, receptor and the glycans they are attached and subsequently avoid recognition, for instance, sialylation of the receptor β1-integrin was found to protect cancer cells against, galectin-mediated apoptosis.[52,53] In contrast, they are crucial ligands for pathogenic receptors or cell surface lectins. For instance, Sias recognition by influenza virus receptors is a necessary step for infection.[54] Importantly, Sias play a crucial role in cell recognition and trafficking.[55,56] More emphasis will be placed on Sias' functions in the cancer context in the topic 1.3.5.

1.2.3. Sialyltransferases

As mentioned before, the transfer of Sias from CMP-Sias to the newly synthesized glycoconjugates is catalyzed by STs, a family of Golgi membrane-bound glycosyltransferases. Eukaryotic STs localization within the Golgi compartment is regulated by specific signals.[48] STs enzymes present conserved aminoacid sequence motifs between each other, whereas the biggest one with 48/49 aminoacids (sialylomotif) seems to represent the binding site to the donor substrate, namely CMP-Sias.[57] STs are divided in four groups based on their donor and acceptor specificity, the nature of the accepting terminal monosaccharide and the type of linkage formed, namely β-galactoside α2,3 sialyltransferase (ST3Gal), β-galactoside α2,6 sialyltransferase (ST6Gal), N-acetyl-galactosamine α2,6 sialyltransferase (ST6GalNAc) and α2,8 sialyltransferase (ST8Sia). The acceptors of the Sias can be galactose or GalNAc residues, via C3 or C6 linked to the C2 of the sialic acid (α2,3 and α2,6 sialylation, respectively), and even other Sias (polysialic acids) through a linkage between the C2 and C8 (α2,8 sialylation).[58] The first family, ST3Gal, include six different subfamilies that catalyze the transfer of Sia residues to a terminal galactose through a α2,3 linkage; the two members of the ST6Gal family identified mediate the transfer of Sia residues by an α2,6 linkage to a

galactose β1-4 N-acetylglucosamine (Galβ1-4GlcNAc) found as a free disaccharide or as the terminus of a glycan; ST6GalNAc family contains six different subfamilies responsible for the transfer of the Sias to GalNAc through a α2,6 linkage; and finally, the last family, ST8Sia which contains also six subclasses that catalyze the transfer of Sias to other terminal Sias in a α2,8 linkage.[59–61] The products from all these reactions can be experimentally recognized by specific lectins. In the context of this thesis, it is important to highlight the *Sambucus nigra* lectin (SNA) isolated from elderberry bark. This lectin preferentially binds to galactose-linked Sia by an α2,6 linkage (ST6Gal product) and by α2,3 linkage to a lesser degree (ST3Gal product).[62] Taking into consideration the scope of this thesis, we will focus on the ST6Gal family, namely the subfamily ST6Gal-I will be further explored in the next topic.

1.2.4. β-galactoside α2,6-sialyltransferase 1

ST6Gal-I (E.C.2.4.99.1) is involved in the addition of α2,6-linked Sia residues to Galβ1-4GlcNAc on N-glycans. In mammals, ST6Gal-I shows a broad tissue distribution with different patterns of expression, with a particular high expression in the liver.[63] Human ST6Gal-I is localized within the Golgi complex and consists of a type II membrane protein (C-terminal oriented to the lumen) with a sequence of 406 aminoacids with two potential N-glycosylation sites.[64,65] At the transcriptional level, both in normal and in cancer tissues, the single ST6Gal-I gene expression is regulated by different promoters.[66] The ST6Gal-I liver expression is accomplished and dependent on the promoters P1 and P3, the first one being the main responsible for the liver ST6Gal-I transcripts. The P3 promoter is constitutive and non-specifically expressed within different tissues. Besides P3, three other promoters mediate the ST6Gal-I expression in B cells, namely, P2a, P2b and P2c. Finally, the ST6Gal-I expression in lactating mammary gland was found to be mediated by P4.[67] The differential usage of promoters and alternative modes of exon splicing originate messenger ribonucleic acid (mRNA) transcripts with divergent 5'-untranslated regions.[68] Three transcripts have been found so far, the Y+Z form that is thought to represent the basal expression, the hepatic form which represents the major liver transcript, and yet another B cell specific.[66] The generation of ST6Gal-I-deficient mice has already shown the importance of this enzyme activity for the immune system. They have an impaired immune response in terms of B cell activation and humoral activity, alterations in thymopoiesis, granulopoiesis and in eosinophil and DC profiles.[69–73] Also, some ST6Gal-I products constitute ligands for certain lectins, for instance the mature B cell surface marker CD22.[74] In contrast, α2,6 sialylation is responsible for blocking the recognition of glycans by β-galactoside-containing lectins (galectins).[52]

Besides the membrane bound form, ST6Gal-I can also be found in its soluble form after proteolytic cleavage by beta-site-amyloid precursor protein-cleaving enzyme 1 (BACE 1), also expressed in the Golgi apparatus of several cell types.[75] Soluble ST6Gal-I retains both folding and

catalytic activity, being biologically functional. However, the soluble form recognized different asialoproteins equally in contrast to the membrane form that recognized them in a different manner, suggesting lower acceptor specificity derived from truncation of the full-length ST6Gal-I.[76]

1.2.5. Biological roles of ST6Gal-I soluble form: extrinsic glycosylation.

It is well known that glycosyltransferases, including ST6Gal-I, are present extracellularly in the bloodstream.[77] However, the possibility of such enzymes to act as extracellular glycan-modifying enzymes seemed unlikely because soluble ST6Gal-I glycosylation was not efficient as it seemed that there were not enough sugar donor substrates available.[78] This concept is now being revised due to recent observations. Firstly, circulatory ST6Gal-I (already known to be a positive APP, but with an unclear contribution[79]) was found to be a regulator of inflammatory cells production.[71,80,81] Secondly, α2,6 sialylation of bone marrow hematopoietic stem cells is mediated by extracellular ST6Gal-I as these types of cells does not express the enzyme endogenously.[82] Besides, ST6Gal-I-deficient mice revealed that IgG sialylation can occur in the bloodstream in a B cell secretory pathway-independent manner.[83] Importantly, platelets were shown to be efficient sources of glycosyltransferases and CMP-Sia donor substrate upon activation and capable of driving the extrinsic sialylation *in vitro*.[84,85] More recently, Manhardt *et al.* 2017 have shown that extrinsic sialylation does occur to a significant extent in a platelet function-dependent manner, although this process is not constitutive.[86] All these reports point towards an alternative pathway to generate or remodel cell surface glycans that consequently influence the function of the immune system.

1.3. Cancer

Cancer is one of the leading causes of mortality worldwide, with approximately 8 million cancer-related deaths and 14.1 million new cases in 2012.[87] In 2025, it is expected that more than 20 million new cases will arise showing the major impact of cancer globally. Every cancer type arises from genetic mutations or epigenetic alterations derived from chemical (tobacco, alcohol, among others), physical (radiation) or biological (infection, chronic inflammation) carcinogenics.[87] Malignant transformation is accompanied and characterized by disruption of genetic information that dictate the characteristics of a normal cell and up or downregulated expression of multiple genes that enhance tumor cells proliferation capacity and their survival, for instance, by the downregulation of genes involved in the apoptosis.[88] The features of cancer will be elucidated in the point 1.3.1.

1.3.1. Cancer Hallmarks

Nowadays, there are a set of features that distinguish cancer cells from normal ones acquired during tumor development, also known as hallmarks of cancer (Figure 1.3). While normal cells proliferation is modulated by exogenous growth and antigrowth factors, not only tumor cells have oncogenes that mimic these signals and the ability to generate their own growth molecules (sustained

proliferative signaling), but also are able to overcome antigrowth signals. These properties, along with the capability to evade apoptosis, confer tumor cells an unlimited replicative potential.[89] This uncontrolled proliferation is sustained by an adaptation mechanism to support the altered biosynthetic requirements. In this situation, known as Warburg effect, there is an up-regulation of autophagy to provide recycled metabolic intermediate and the activation of the alternative aerobic glycolysis metabolic profile, i.e. the conversion of glucose to lactate in the presence of oxygen.[90] Tumor expansion is achieved due to the angiogenic deregulation and allows tumor cells to have access to oxygen and nutrients necessary for cell growth, although the hypoxic niche of tumor growth metabolism is supported by the Warburg metabolic shift.[89,90] Alterations in the expression of cell-cell adhesion molecules and integrins (responsible for cell-extracellular matrix (ECM) adhesion), as well as activation of ECM-degrading proteases are factors that enable tumor growth and metastatic capability. Besides, there are two emerging hallmarks that might be included referring to the capabilities of deregulating cellular energetics and evading immune response. Besides, genome instability and tumor-promoting inflammation are considered two enabling characteristics that allows the acquisition of all these hallmarks.[89]

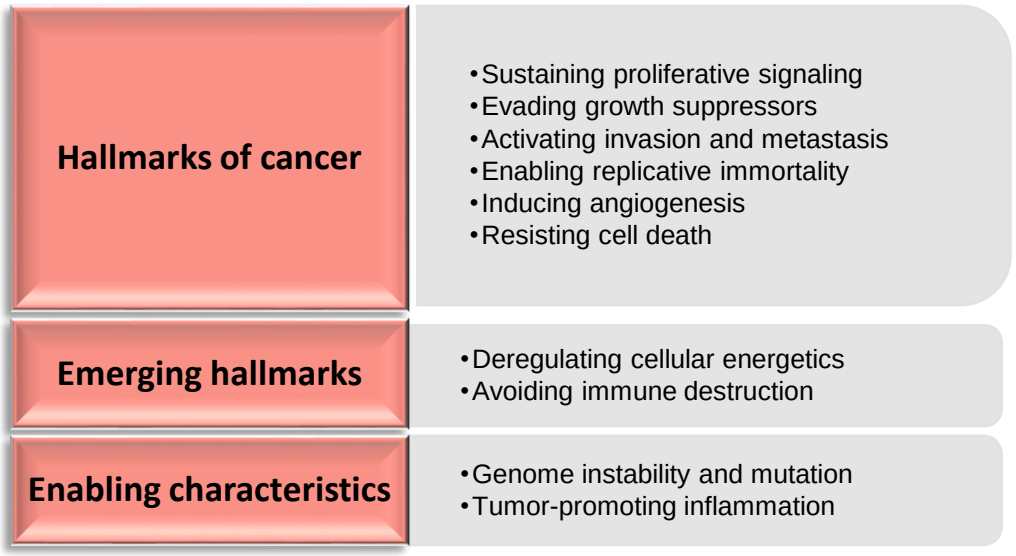

Figure 1.3 – Hallmarks, emerging hallmarks and enabling characteristics of cancer. Adapted from Hanahan and Weinberg 2011.[89]

1.3.2. Cancer evasion of the immune response

The immune system (already depicted in the topic 1.1) has three important roles in the prevention of cancer. Firstly, as previously mentioned, immune cells can eliminate viral infections that can induce virus-induced tumors. Secondly, the destruction of pathogens and resolution of the inflammatory state will prevent tumors promoted by an inflammatory microenvironment. Finally, the

immunological system can identify tumor-specific antigens or stress-induced molecules and proceed with tumor cells elimination (tumor immune surveillance).[91] However, tumors are still capable of developing in spite of the existence of this protective mechanism in immunocompetent individuals. Cancer cells mediate the production of chemokines that recruit $CD4^+FOXP3^+CD25^+$ T cells (also known as regulatory or suppressive T cells) with an enhanced suppressive activity to downregulate the induction or proliferation of T_C cells to the tumor microenvironment.[92] Other cytokines/chemokines/factors present in the tumor microenvironment can act themselves as suppressive factors or even promote cancer growth and progression. Cancer cells use downregulation of the antigen presentation machinery, including MHC-I, decreasing the expression of tumor antigens and thus escaping recognition and elimination by the T_C cells. Tumor cells also have the advantage to fail to express costimulatory molecules and to downregulate death receptors, which induce anergy or tolerance in T cells and prevents death-ligand-mediated cell killing.[93] Besides, they are capable of deviating the immune response from T_H1 to T_H2, a state that has been proposed to facilitate tumor growth.[93,94] Also, there is evidence that tumor cells can eliminate tumor-specific T_C cells through apoptosis.[93] Finnaly, the glycosylation also play a important role in cancer immune evasion being the aberrantly high expression of Sia in tumor cells' surface (topic 1.3.3) envolved in antigen masking and prevention of recognition by the immune system.[95]

1.3.3. Glycosylation and Cancer

The transformation of a normal cell into a cancerous one is accompanied by well-documented changes in the cellular glycosylation patterns. These glycan changes can vary from hypo- or hyper-expression of certain structures, the presence of incomplete or truncated structures, accumulation of precursors or even appearance of novel structures, such as the highly fucosylated Lewis antigens, $Lewis^{a/b}$ and $Lewis^{x/y}$.[96] The specific glycosylation alterations that occur are correlated with cancer prognosis once it plays a leading role since cancer progression to metastasis and even signaling.[28,97] One of the main alterations in malignant transformation is abnormal sialylation. Specifically, hyper-sialylation is associated with poor outcome and malignant properties as invasiveness and metastatic potential influencing tumor progression.[98] Indeed, STs and Sias are extremely relevant in cancer because they can prevent cell-cell interaction through charge repulsion effects, promote the bounding to cell adhesion molecules, mask carbohydrate structures avoiding recognition by the immune system and show if a specific signaling pathway has been activated.[99] Three different mechanism have been reported to cause aberrant glycosylation in tumor cells: (1) overexpression or deregulated activity of STs increases the sialylation state of cancer cells and the expression of specific tumor-associated carbohydrate antigens; (2) increase in the metabolic flux in the Sia synthesis pathway in malignancy due to increase of substrate availability or overexpression of the genes involved in this pathway; and (3) decreased expression of endogenous sialidases (enzymes that catalyze the cleavage of Sias from glycans) leading to sialylated glycans accumulation.[100]

1.3.4. Colorectal cancer

Colorectal cancer (CRC) is a major cancer burden being the third type of cancer with more incidence (9.7%) and the fourth responsible for more cancer-related mortality (8.5%), globally.[87] This type of cancer may rise from different causes, namely, genetic heritage, dietary composition or even environmental factors.[87] The development of this type of cancer was proposed to be multi-stepwise by Fearon and Vogelstein 1990 and rely on mechanisms that involve chromosomal instability, microsatellite instability, aberrant deoxyribonucleic acid (DNA) methylation and DNA repair defects.[101,102] It starts with the accumulation of a set of mutations in the normal colon that creates an hyperproliferative benign adenoma. These mutations affect for instance the *RAS* gene and cause the inactivation of the tumor suppressor gene, considering adenomatous polyposis coli. The evolution to a malign carcinoma is related to events like mutations in p53 and gain of the chromosome 8q. The development of the CRC metastatic capacity occurs in 10 years, due to the acquisition of another set of genetic and epigenetic mutations in specific oncogenes and/or tumor suppressor genes as well as the loss of chromosome 8q.[102] The disease heterogeneity at the molecular level of CRC defining aggressiveness and therapy resistance, as well as the different clinical presentation depending on the tumor site show the dangerousness of this type of cancer.[102] Treatment of CRC is typically limited to surgical resection with the possibility of adjuvant chemo or radiotherapy conjugation, however, there is still a high risk of metastasis with a low survival rate due to failure of treatment response.[103] CRC tissue or cells have pronounced differences in glycosylation compared to normal cells, specifically in N-glycans, O-glycans, glycosphingolipids, fucosylation, sialylation and Lewis antigens patterns, that influence tumorigenesis, metastasis, anti-tumor immunity and resistance to therapy.[104] In the context of this thesis, more emphasis will be placed in the altered α2,6 sialylation derived from the activity of ST6Gal-I (topic 1.3.5).

1.3.5. ST6Gal-I and CRC

ST6Gal-I is one of the many glycosyltransferases with altered expression in cancer.[99] Several types of cancer, including CRC, have increased expression or activity of ST6Gal-I and also show higher levels of α2,6 sialylation, which correlates with poor prognosis.[99,105,106] However, the correlation between ST6Gal-I expression and activity is not straightforward and neither are the phenotypic effects. On the one hand, it seems to exist a relationship between ST6Gal-I and malignancy in terms of invasion and metastatic potential. In fact, studies with human CRC cell lines showed that lower ST6Gal-I activity and α2,6 sialylation content diminish the ability of the cells to form colonies and their invasive potential.[107] Moreover, ST6Gal-I is overexpressed in a non-adherent subpopulation derived from an adherent population of SW948 cell line, a 81 year female with Dukes' type C, grade III colorectal adenocarcinoma-derived cell line, which points to the importance of α2,6 sialylation on cellular adhesion properties.[108,109] On the other hand, there is data that contrast with those observations. It has been proposed that such different results rely on the competition with other STs

or substrates whose expression is different from tissue to tissue and even cell line to cell line from the same type of tumor.[99] Besides cell migration and invasion, ST6Gal-I may also play a pivotal role in cell death evasion by preventing Fas or TNF-α-mediated apoptosis.[110,111] More recently, it was suggested that ST6Gal-I promote tumorigenesis and can act as a cell marker and regulator of cancer stem cells, i.e., cells capable of give rise to different types of cells within the tumor.[105] Regarding soluble ST6Gal-I, higher levels of this form in the bloodstream have been correlated with progression and metastasis of CRC, however the possible usage of this enzyme as a biomarker and its biological function in the cancer context remains elusive.[112]

1.4. Congenital Disorders of Glycosylation

Mutations in the genes involved in the glycosylation pathways are the main cause of defective glycan biosynthesis and hypo- or hyper-glycosylation of proteins, which originate Congenital Disorders of Glycosylation (CDG).[35,113] CDG are a rapidly expanding family of rare inherited metabolic diseases with more than 100 different CDG types identified. Each affected glycosylation-related gene gives rise to a different CDG type (Figure 1.4). Since glycans play important roles in all organs and tissues, CDG phenotypic manifestations are mostly multi-systemic, being the nervous system the mainly affected organ[114], but other organs can also be severely compromised, including the liver[115], the skeleton[116], the heart[117] as well as the immune system[118]. Diagnosis encompasses molecular testing, namely isoelectric focusing (or capillary zone electrophoresis) of serum transferrin to identify N-glycosylation deficiencies and isoelectric focusing of serum apolipoprotein C-III to identify O-glycosylation disorders. If still the results are inconclusive enzymatic testing or mutation analysis by whole exome sequencing are executed.[119] The treatment options are limited to few CDG types, being symptomatic and supportive therapies the only way of disease management in the majority of the cases.

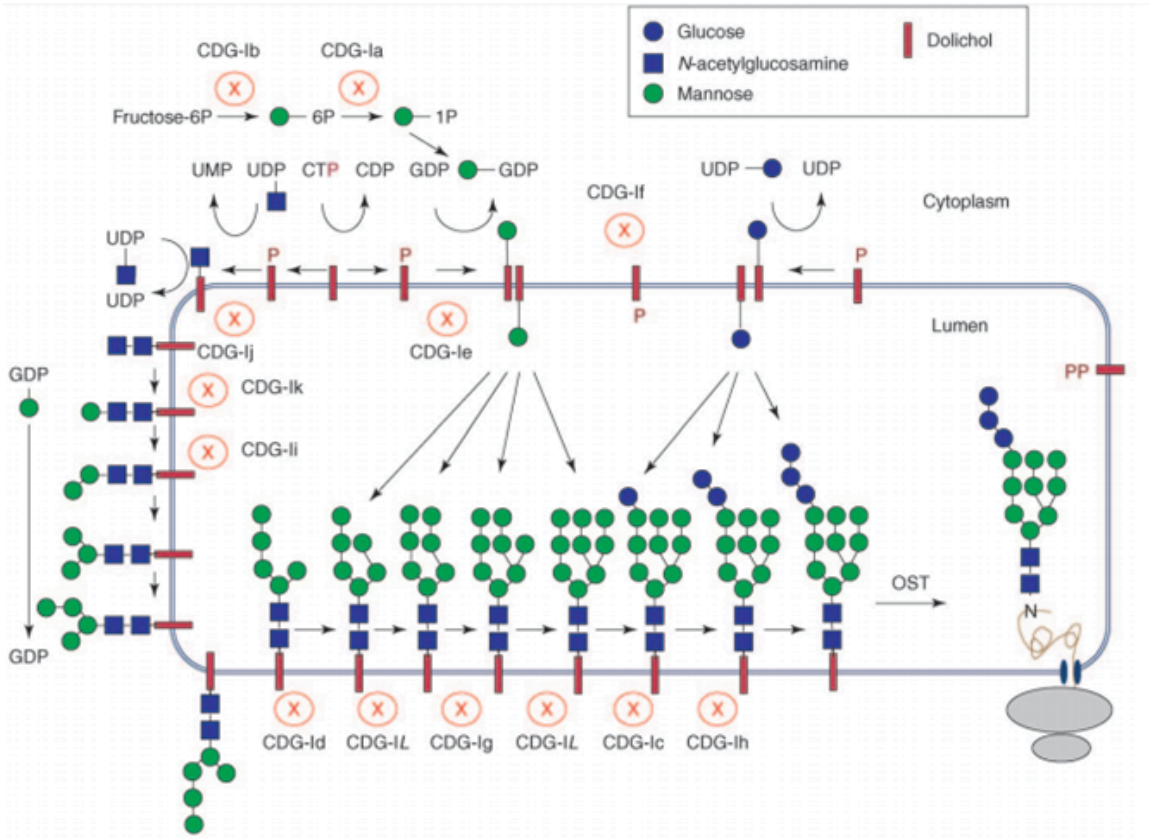

Figure 1.4 - Congenital disorders of N-glycosylation. Each CDG type arises from a mutation in a specific gene that codifies a specific enzyme of the biosynthetic pathway. The different CDGs are represented with the old nomenclature. The new nomenclature was introduced in 2009. From Jaeken *et al.* 2009.[120]

1.4.1. PMM2-CDG

PMM2-CDG (previously known as CDG Ia) is the most frequent type of CDG and is derived from mutations in the *PMM2* gene that codifies the phosphomannomutase 2 (PMM2) enzyme (EC 5.4.2.8).[121] This protein catalyzes the conversion of mannose-6-phosphate in mannose-1-phosphate that is the precursor necessary for the second step of the N-glycosylation biosynthetic pathway. PMM2-CDG has a wide clinical spectrum whereas most common phenotypic features include neurological affectation, failure to thrive, psychomotor retardation, development delay and inverted niples.[122] In the first years of life, the mortality rate is approximately 20% due to cardiomyopathy, liver failure and recurrent severe infections.[122] These and other not so frequent symptoms are common to many other diseases. Although management therapies are available, no curative treatment exists for this CDG type. Moreover, there are no established care guidelines once the natural clinical history of PMM2-CDG has not yet been defined. These factors along with disease severity are the main reasons for the profound impact in patients' and caregivers' quality of life (QoL). In fact, PMM2-CDG patients have been shown to have a significant increase in depression, social problems and somatic complains.[123]

1.4.2. PMM2-CDG immunological involvement

Contrasting to other types of CDG, PMM2-CDG has a minor immunological involvement since only a small set of patients have been reported with immunological issues.[118] However, the mechanisms underlying immunological affectation are yet not well understood. Recurrent and severe infections and fever-triggered stroke-like episodes are the main immune symptoms, whereas the first has been found to affect PMM2-CDG patients throughout childhood and to disappear during adult life.[124] Several pathogens have been identified in the origin of these infections, namely *Pneumocystis carinii*[125], *Streptococcus pyogenes*[126], *Pseudomonas*, *RSV*, *Influenza pneumonia*, *E. Coli*, *S. viridans* and, in same case, vaccination of patients was found to be ineffective or to elicit illness[127]. Although hypogammaglobulinemia has been found to occur in some patients, no correlation was found between low immunoglobulin levels and infections in 15 PMM2-CDG patients.[128] The recruitment of neutrophils in response to a stimuli (chemiotaxis) was also found to be decreased in few cases.[127] Blood levels of certain cytokines were found altered in certain conditions showing an altered inflammatory response, maybe due to an overload of underglycosylated proteins in the ER.[129] Other rare clinical presentations were also reported, namely thrombocytopenia and hyperferritemia in the absence of an infection and with an unexplained macrophage activation.[130] Blood proteins and cell surface glycoproteins related with the immune response were also found to be decreased or altered in terms of mass/charge or α2,6 sialylation.[118]

1.5. Patient Reported Outcome Measures and Observer Resported Outcome Measures

Patient-centricity is a recent approach in clinical research and practice.[131] In a patient centric approach, the input of a patient about the manner in which his physical, emotional and social well-being are affected by his/her own condition or treatment (Health Related Quality of Life – HRQoL) is achieved through the use of patient reported outcomes measures (PROMs) and observer reported outcomes measures (ObsROMs).[132] PROMs are direct reports from patients about their health condition without any interpretation by clinicians or any other health professional, and can be measured using validated questionnaires.[133] As for ObsROMs, these are reports made by caregivers or any non-medical person or professional, who is in direct contact with the patient. ObsROMs are used to evaluate symptoms and functioning in young, too ill or cognitively impaired patients, who are incapable of doing self-reports.[134] These measures have been increasingly employed in clinical trials research as primary endpoint measures, as well as to measure effectiveness and safety of a drug or therapy.[132] While PROMs and ObsROMs have been frequently used in common human disorders, the scenario is not so bright in the rare diseases field.[135] In fact, there are a number of challenges in implementing and developing these measures due to disease heterogeneity, availability of suitable measures, patient recruitment and selection of appropriate data collection methods.[134] In spite of this, some examples of successful use of PROMs in rare diseases have been registered, in particular in academia, PRO methodologists, and patient organizations collaborative approaches.[134,136]

1.6. Introduction to the aims of this thesis

Glycosylation is known to be crucial in many biological processes and altered glycosylation is related with many diseases influencing phenotypic maniphestations.[37] Regarding the immunological system, glycosylation is essential for events like cellular recognition, signaling, lymphocyte development and lymphocyte homing.[40,42–47]

Recent studies are highlighting the immunomodulatory role of cell surface Sias. Indeed, observations from our group suggested that sialidase treatment on human monocyte-derived DCs (moDCs) increase the expression of maturation markers, their genetic expression of pro-inflammatory cytokines and the priming and activation of T lymphocytes. This increase in functionality was attributed to α2,6 sialic acid removal, as shown by the analysis of ST6Gal-I$^{-/-}$ mice DCs.[137] A number of STs are in the origin of cell surface sialylation. Specifically, the α2,6 sialylation is catalyzed by the ST6Gal and the ST6GalNAc families that differ in terms of their Sias acceptor specificity, galactose and GalNAc, respectively.[59] Regarding ST6Gal-I, besides its membrane-bound form, this enzyme presents a cleaved biologically functional secreted form, which points towards an alternative pathway of extrinsic glycosylation that is able to modulate the cell surface glycosylation.[75,76,80,84,85] Our group is part of a consortium with 15 European partners in the fields of glycobiology, glycoimmunology and biomarker research that aims to understand the structure-function relationship of glycans in CRC for finding improved diagnostic and prognostic biomarkers, and pave the way for novel therapeutic targets. Thus, a part of this master project aims to explore the aberrant glycosylation of CRC cells and its implications in the immune system, contributing to the ultimate goal of the consortium. CRC has been found to upregulate the expression and activity of ST6Gal-I and subsequently increase the levels of α2,6 sialylation, which influences malignancy in terms of invasive and metastatic potencial.[138] Therefore, we hypothesized that CRC cells possibly secret ST6Gal-I and this enzyme can modify the extrinsic glycosylation of immune cells and modulate their immune function.

CDG patients present immunological affectation with the severity of immunological dysregulation being dependent on the CDG type.[118] The importance of glycosylation in the immune system has made us hypothesize that the defective glycosylation observed in CDG influences the function of immune cells. PMM2-CDG patients' clinical manifestations include minor immunological involvement, such as recurrent and severe infections during childhood and fever-triggered stroke-like episodes.[124] Despite the wide clinical spectrum of PMM2-CDG patients, only management therapies are available for this type of CDG, as well as for the great majority of CDG types and other rare diseases. We are part of the international network CDG & Allies – PPAIN dedicated to research on rare diseases, with especial dedication to CDG. Specifically, we integrate the working group CDG & Glycoimmunology that aims to increase our understanding about the mechanisms behind the altered immune responses in CDG patients. This line of reseach will contribute to find targets for CDG treatment.

PMM2-CDG is a very debilitating condition and have a profound negative impact in patients' and caregivers' QoL, especially due to lack of treatment, lack of care guidelines and the disease heterogeneity.[134] This reality made us get envolved in the working group of CDG & Patient Reported Outcomes dedicated to understand CDG symptoms, the family experience and the impacts of this illness. Since the increased efforts to find curative treatment for CDG are now highlighting the necessity of specific patient reported outcomes assessment tools to evaluate the QoL as a primary endpoint in clinical trials, to find, adapt and/or validate suitable existing PROMs or ObsROMs would fulfill the existing gap and facilitate clinical outcome assessments.

To achieve our goals, the aims of this thesis consisted in to (1) study the aberrant glycosylation of CRC cells and its modulation of the immunological system, concerning CRC expression and secretion of ST6Gal-I and if this enzyme is able to extrinsically sialylate DCs affecting its phenotype and immune function; (2) create a PMM2-CDG cell line to provide a new line of research to unravel immunological aspects of PMM2-CDG patients and evaluate their immune response to PMA and Ion mitogenic stimulation in comparison to a healthy sibling and (3) review the existing PROMs and ObsROMs to assess CDG patients' and caregivers' QoL.

2. Materials and Methods

2.1. Cell culture

2.1.1. Isolation, cryopreservation, thawing and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) for optimization studies were isolated from buffy-coats of male healthy volunteers between 18 and 60 years old, provided and ethically approved by the Portuguese Blood Institute. The blood samples from the 35 years old PMM2-CDG patient donor enrolled in this study and the corresponding 39 years old healthy sibling (control without mutations in the *PMM2* gene) were donated with consent for experimental purposes. PBMCs were isolated by density gradient centrifugation using Biocoll (Merck Millipore). 30 mL of buffy-coat was centrifuged at 1100 g, for 10 minutes, at room temperature (RT), without brake, and the PBMCs as well as the plasma were collected to a 50 mL falcon to which phosphate buffered saline (PBS) (Appendix 1) was added up to 40 mL. After mixing, the cell suspension was divided and slowly added to 12 mL of Biocoll at RT, and centrifuged at 1100 g for 30 minutes, without brake. The interface ring was collected to a new tube, diluted with PBS up to 40 mL and the cell suspension was centrifuged at 1100 g for 10 minutes at RT. The supernatant was discarded and the pellet resuspended in 10 mL of PBS. Cells were counted under the optic microscope using a Neubauer chamber. The cell suspension was centrifuged at 400 g for 10 minutes at RT for platelet removal. The PBMCs were frozen at -80º in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamine, 1% (v/v) non-essential aminoacids and 1% (v/v) sodium pyruvate (complete RPMI – cRPMI), as well as 10% dimethyl sulfoxide. When thawed, PBMCs were cultured in cRPMI in an incubator at 37ºC with a humidified atmosphere and 5% CO_2, except in the first 18 H in which they were maintained in cRPMI with 20% FBS before any other procedure. Both RPMI and supplements were from Gibco®, Life Technologies.

2.1.2. Culture of CRC cell lines

Four different CRC cell lines were used, specifically SW48 NC, SW48 ST6, SW948 NC and SW948 ST6. SW48 and SW948 cell lines were transduced with the lentiviral vector pLenti6/V5-Directional TOPO containing the cDNA of the gene that encodes for human ST6Gal-I to originate SW48 ST6 and SW948 ST6 cell lines, and kindly provided by Fabio Dall'Olio and co-workers. Similarly, the same original cell lines were transduced with an empty vector (mock) to give raise to SW48 NC and SW948 NC cell lines.[139] CRC cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin/Steptomycin and 1% (v/v) Glutamine (complete DMEM - cDMEM) in an incubator at 37ºC with a humidified atmosphere and 5% CO_2. DMEM and supplements were from Gibco®, Life Technologies. The medium was changed every 3 days and the cells were sub-cultured every 8-9 days by incubating them with trypsin-

ethylenediaminetetraacetic acid (EDTA) (Gibco®, Life Technologies) for 5-10 minutes, followed by the addition of 3 times the volume of trypsin of pre-warmed medium and a centrifugation at 200 g, during 5 minutes at RT.

2.2. Immunomagnetic separation

In order to isolate $CD3^+$ cells, thawed PBMCs were centrifuged and resuspended in 80 µL of beads buffer (Appendix 1) and 20 µL of CD3 magnetic microbeads (Miltenyi Biotec) for each $3x10^7$ cells. After incubating for 30 minutes at 4ºC, cells were washed with beads buffer and centrifuged at 900 g for 10 minutes at RT. The pellet was resuspended in 2 mL of beads buffer and added to a LS column assembled to a MidiMACS Separator in a MACS Multistand (Miltenyi Biotec), previously activated with 3 mL of beads buffer. After the column was washed three times with 3 mL of beads buffer, the column was removed from the action of the magnetic field, and the positive fraction was eluted using a plunger. The $CD3^+$ cells were counted and cultured in cRPMI until further use.

2.3. Assay to test the specificity of the anti-ST6Gal-I antibody.

To test the specificity of the anti-ST6Gal-I Ab, purified ST6Gal-I (a kind gift from Joseph Lau) was used. To obtain lysates, tissues from ST6Gal-I deficient (SG)[69] ant wild type (WT) mice (obtained from Consortium for Functional Glycomics) were defrosted and washed with PBS to remove the excess of blood. After cutting the tissue in small pieces using a scalpel as quickly as possible to prevent degradation by proteases, ~300 µL of ice cold IP Lysis/Wash Buffer (Thermo Scientific) complemented with protease inhibitors (Roche) per ~3 mg of tissue was added and homogenized. The pellet pestle (Sigma-Aldrich) was rinsed with another volume of lysis buffer and the suspension was incubated during 20-30 minutes with periodic vortexing every 5 minutes. A centrifugation at 17000 g during 20 minutes at 4ºC was preformed to remove cell debris, the protein concentration of the supernatant was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) following manufacturer instructions, and the supernatant was saved at -80ºC until further use. The specificity for ST6Gal-I was assessed using the Sodium Dodecyl Sulfate-Polyacrylamide Gel Eletrophoresis (SDS-PAGE) and Western Blot (WB) techniques as described in the point 2.6.

2.4. Assay to test the expression of ST6Gal-I in CRC cell lines.

To test if the CRC cell lines express ST6Gal-I, cells were grown until confluency and cell lysates were obtained by incubating cell pellets during 20-30 minutes with IP Lysis/Wash Buffer (Thermo Scientific) complemented with protease inhibitors (Roche) with periodic vortexing followed by a centrifugation at ~13000 g during 10 minutes to remove cell debris. Protein concentration was accessed using Pierce BCA Protein Assay Kit (Thermo Scientific). The presence of ST6Gal-I in the cell lysates obtained was assessed using the SDS-PAGE and WB techniques as described in the point 2.6.

2.5. Assay to test the secretion of ST6Gal-I by CRC cell lines.

Two different protocols were attempted to assess ST6Gal-I secretion. In the first one, cells were cultivated in cDMEM with 2% FBS with a cell density of 1×10^6 cells/mL and supernatants were recovered after 24 H, 48 H, 72 H and 96 H. In the second one, cells were cultured in cDMEM + 10% FBS with a cell density of 1×10^6 cells/mL. When confluency was achieved, the medium was changed to cDMEM without serum. Supernatants were recovered after 24H, 48H and 72H. A 12 times concentration of the supernatants was done using Amicons Ultra 0.5 mL centrifugal filter devices (Merck Millipore) by a centrifugation at 14000 g during 10 minutes at RT. The presence of ST6Gal-I in the supernatants was assessed using the SDS-PAGE and WB techniques as described in the point 2.6.

2.6. SDS-PAGE and WB

SDS-PAGE and WB were performed to test the Ab specificity used in this study (point 2.3.) and to test the presence of ST6Gal-I whether in cell lysates (point 2.4.) or in supernatants (point 2.5.). Samples were loaded in 4% stacking (Appendix 1) and 12% resolving gel (Appendix 1) and the electrophoretic run was performed in running buffer (Appendix 1) at 80V-100 V for 30 minutes, and at 150 V until the end of the run. The proteins transfer into a polyvinylidene fluoride (PVDF) membrane was performed in transfer buffer (Appendix 1) at 400 mA for 1 H and the membrane was blocked with a 5% non-fat milk powder (VWR®) diluted in tris buffered saline with 0.1% Tween-20 (TBS-T 0,1%) during 1 H at RT. Then, the membrane was probed with 10 µg/mL of anti-ST6Gal-I Ab diluted in TBS-T 0,1% (Appendix 1) overnight at 4ºC. After washing with TBS-T 0,1%, a horseradish peroxidase (HRP) goat anti-mouse Ig Ab (BD Pahrmingen™) at a 1:2500 dilution in TBS-T 0,1% was added and incubated at RT for 1 H. After washing, detection was performed using Lumi-Light Western Blotting Substrate (Roche), mixing the two reagents in a 1:1 proportion and incubating the membrane during 1 minute at RT. Finally, revelation was achieved exposing the membrane to Amersham Hyperfilm ECL (GE Healthcare Life Sciences) using Corestream® Kodac® autoradiography GBX developer/replenisher and Corestream® Kodac® autoradiography GBX fixer/replenisher (Sigma).

2.7. Immortalization of PMM2-CDG-PBMCs cells

PMM2-CDG-PBMCs cells immortalization was a service performed by Abm®. Cells were plated in 6-well plates with 30-40% cell density, 24 H before infection with 2 mL per well of Lenti-GFP containing Epstain Barr Virus (EBV) (G229 - 10^6 IU/mL) in the presence of 10 µg/mL polybrene and ViralPlus (G698) at 1:100 ratio. 6-8 H later, viral supernatant was removed from the wells and 2 mL of fresh viral supernatant was added per well (supplemented with polybrene and ViralPlus) for a second infection overnight. After infection, the viral supernatant was removed, and the cell were cultured in cRPMI supplemented with 50 ng/mL hIL2 at 37ºC with 5% CO_2). After 72 H incubation, the cells were sub-cultured into 2x100 mm dishes with the selection drug neomycin. Antibiotic

containing medium was replaced every 3-4 days using the same antibiotic concentration. 10-15 days after selection, clones were picked for expansion and positive ones were screened. Morphology was assessed with optical microscopy.

2.8. Flow Cytometry

Flow cytometry is a technique that allows a multi-parameter analysis of single cells in a suspension (more information in Appendix 2, topic 6.2.1.). This technique was employed in this work to evaluate α2,6 sialylation (point 2.8.1.), PBMCs populations (2.8.2.) and to assess cell proliferation (2.8.3.), using the Attune® Acoustic Focusing Cytometer (Applied Biosystems).

2.8.1. SNA staining

To evaluate the differences of cell surface content in α2,6 sialylation, SW48 NC, SW48 ST6, SW948 NC and SW948 ST6 were pelleted by centrifugation at 300g, during 5 minutes at RT and resuspended in 500 µL FBS-containing medium. The cells were stained with 2 µL of fluorescein isothyocyanate (FITC)-conjugated SNA lectin for 5-10 minutes on ice. Then, cells were diluted in PBS 1x and centrifuged in the same condition as before. The cells were analyzed right away after resuspended in 300 µL of PBS and at least 10000 events were acquired.

2.8.2. Evaluation of PBMCs populations

Before cryopreservation, PMM2-CDG and control cells were stained with several Abs according to Table 2.1 to access for cell surface markers. To each tube containing 100000 cells in 100 µL of PBS, 3 µL of each Ab were incubated on ice during 15 minutes in the dark. The cells were washed with 1 mL of PBS, centrifuged at 200 g during 5 min at 4ºC and the pellet was resuspended in 500 µl of PBS. The cells were analyzed right away and at least 10000 events were acquired.

Table 2.1- Scheme of the conditions used in the flow cytometric evaluation of PBMCs populations. Legend: FITC - fluorescein isothyocyanate, PE – phycoerythrin, Apc – allophycocyanin.

Tube	Staining	Channel	Goal
1	Unstained (US)		Control
2	CD4 FITC	BL-1	Compensation
3	CD14 PE	BL-2	Compensation
4	CD3 Apc	RL-1	Compensation
5	CD4 FITC CD45 PE CD3 Apc		Cytotoxic and helper T cells
6	CD14 PE		Monocytes
7	CD45 PE CD19 Apc		B cells

2.8.3. Evaluation of the PBMCs' proliferation capacity after mitogen stimulation

To study cells proliferative capacity, two different CellTrace™ Cell Proliferation Kits (Invitrogen™, Life Technologies) were used. All optimization steps were performed with cryopreserved cells with at least one year old. 24 H after thawing, the cells were centrifuged at 300 g during 5 minutes at RT (the following steps of centrifugation were performed in the same conditions) and resuspended in PBS. After centrifuging, cells were counted and the necessary number of cells were resuspended in 2 mL of PBS and a 0.2 µM CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) solution was added in a 1:1 proportion, slowly and directly into the tube walls, for a final concentration of 0.1 µM. After gently mixing, the suspension was incubated during 10 minutes at 37ºC in the dark. To remove the free dye in solution, 10 mL of pre-warmed PBS with 2% FBS were added and incubated for 5 minutes at 37ºC, in the dark. After centrifugation, the cells were resuspended and cultured in pre-warmed cRPMI media and incubated for 18 H before any other procedure to allow acetate hydrolysis to occur. Once CFSE was found to affect cell viability, CellTrace™ Far Red Cell Proliferation Kit (Invitrogen™, Life Technologies), with lower cell toxicity at working concentrations, was used following the same experimental conditions, except for CellTrace™ Far Red reagent concentration. Two different concentrations of this reagent were tried: specifically, the recommended concentration 1 µM and 0.05 µM. Labeled PBMCs at a concentration of $1x10^6$ cells/mL were stimulated with several combinations of phorbol 12-myristate 13-acetate (Sigma) and Ion (Sigma) and cultured in a 96-well round bottom plate (Costar). PMA concentrations were limited to 20 nM, 10 nM or 1 nM and Ion concentrations varied between 1 µM, 0,5 µM, 0,25 µM and 0,125 µM. The medium was supplemented with human IL-2 (Miltenyi Biotec) at a final concentration of 1 ng/mL to provide T cell survival signals. Cells were collected after 1, 2 and 3 days. Cell fluorescence was collected and the data were analyzed using Attune cytometric software and ModFit LT 3.2 software (Verity Software House, Topsam, ME), allowing to assess the cell proliferation index, which represents the average number of cells that were originated from a single cell of parental generation. The parental generation was set based on the analysis of data obtained from the cells maintained 18 H in culture after staining.

2.9. Evaluation of cytokines by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is an analytic biochemistry assay to detect and quantify the presence of a substance such as proteins, Abs or hormones, in a liquid sample. The relative quantification of IFN-γ was assessed resorting to the ELISA kit (Immunotools) following the manufacturer's instructions but with all volumes cut to half as previously optimized by the group. The wells of a high biding 96-well plate (Costar) were coated with 50 µL of capture Ab, previously diluted 1:100 in PBS, and incubated overnight at 4ºC. After the incubation, the capture Ab solution was removed, 200 µL of blocking buffer (Appendix 1) were added to every well and the plate was incubated for 60 minutes at RT. The standards of each cytokine were prepared with a 1:2 serial dilution from 4000 pg/mL to 0 pg/mL with blocking buffer. After blocking buffer removal, 50 µL of each

standard and sample were added to their respective wells in duplicate and incubated at RT for 2 H. The plate was washed five times using 200 µL of washing buffer (Appendix 1). 50 µL of biotinylated detector Ab, previously diluted 1:1000 in blocking buffer, were added to each well and incubated for 30 minutes at RT. After another series of five washing steps, 50 µL of TMB substrate at RT were added to each well and the plate was incubated for at least 30 minutes in the dark until optimal color development. The reaction was stopped with 25 µL of 4M HCl and the optical density was read at 450 nm on a SpectraMax 190 Microplate Reader (Molecular Devices) and the data were acquired using the SoftMax Pro software (version 6.4.). The results were analyzed using Microsoft Excel and GraphPad Prism (version 6).

2.10. Real-Time Quantitative Polymerase Chain Reaction

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) is an alternative to the conventional PCR technique that allows the quantification of the amplified nucleic acid as the reaction occurs (more information in the Appendix 2, topic 6.2.2.). In this project, RT-qPCR was used to evaluate the efficiency of the PMM2-CDG-PBMCs immortalization (topic 2.10.1.) and to measure cytokine genetic expression (2.10.2.)

2.10.1. Evaluation of the immortalization process

The analysis of the immortalization process was performed by ABM services. RNA was extracted from EBV-PBMCs using TRIzol reagent and cDNA was synthesized by reverse transcription. For the RT-qPCR reaction, specific EBV primers were mixed with 1 µL of 0.1 µg/µL of cDNA template. The EBV forward and reverse primers sequences consisted in 5' AAA CCT CAG GAC CTA CGC TGC and 5'AGA CAC CGT CCT CAC CAC, respectively. In this experiment, the SYBR Green technology was used, resorting to the Step One™ Real-Time PCR instrument with a ROX passive reference dye. This inert dye is used to normalize the variations between wells due to pipetting errors or even instrument limitations.

2.10.2. Evaluation of cytokine gene expression

For the analysis of the genetic expression of the cells after mitogen stimulation, ribonucleic acid (RNA) extraction was performed following its conversion to cDNA. RNA extraction was achieved resorting to GenElute Mammalian Total RNA Miniprer Kit (Sigma Aldrich). The cell lysis solution was prepared by adding β-mercaptoethanol (Sigma) to the commercial lysis solution in a 1:100 proportion. 250 µL of this mixture were added to each sample and cells were lysed by throughout pipetting. The lysate was filtrated resorting to a filtration column by centrifugation at 12000 g during 2 minutes as 4ºC (all the next centrifugation steps were performed in the same conditions). 250 µL of 70% ethanol were added to the filtered lysate, throughly mixed and added to a binding column whereas the RNA stayed attached after centrifugation. This column was washed with 250 µL of washing solution 1 by

centrifugation. The DNAse I solution was prepared by mixing DNAse I with digestion buffer in a 1:8 proportion and 80 µL of this solution were added to each column. After incubation for 15 minutes at RT, the columns were washed once with 250 µL of washing solution 1 and twice with 500 µL of washing solution 2 by centrifugation. The remaining ethanol was removed by centrifugation of the column without any volume. The column was placed into a clean collection tube and RNA was eluted with 55 µL of elution solution by centrifugation. The flow-through was collected, added again to the binding column and centrifuged to elute the remaining RNA. The eluted and purified RNA was immediately converted into cDNA.

The conversion of RNA to DNA was performed resorting to the High-Capacity cDNA Transcription Kit (Applied Biosystems) on the basis that random primers will bind to the RNA allowing the reverse transcriptase enzyme to execute the transcription. 50 µL of each sample of purified RNA were added to a PCR tube (VWR). 50 µL of a mix with the necessary reagents for the conversion was added to each tube. This mix was constituted by 10 µL of buffer 10x, 10 µL of random primers 10x, 4 µL of 100 mM deoxynucleoside triphosphates (dNTPs), 7,5 µL of reverse transcriptase and 18,5 µl of water molecular biology (NZYTech) for sample. RNA conversion was achieved using the program described in Table 2.2, on a Programmable Thermal Controller PTC-100™ (MJ Research), and cDNA were stored at -20ºC.

Table 2.2 - cDNA synthesis PCR program

	Step 1	Step 2	Step 3	Step 4
Temperature (ºC)	25	37	85	4
Time	10 min	120 min	5 sec	∞

For the RT-qPCR, TaqMan chemistry was used. The reaction was carried out in 0,1 mL PCR tubes (Simport) in a total volume of 10 µL. The reactions were prepared in duplicate by adding 3 µL of cDNA, 5 µL of TaqMan Fast Universal PCR Master Mix 2x and 2 µL of probe (Applied Biosystems). The probes included β-actin and glyceraldehyde 3-phosphate dehydrogenase (GADPH) as the endogenous controls, TNF-α and IFN-γ. For this experiment, Rotor-Gene 6000 Series (Corbett) with the conditions described in Table 2.3 were used.

Table 2.3 - RT-PCR reaction conditions.

	Cycle 1	40-50 cycles	
Temperature (ºC)	95	95	60
Time (seconds)	20	3	30

Gene expression was assessed by the C_T method.[140] The C_T values were determined using the Rotor-Gene 6000 series software (version 1.7) and further calculations were performed using Microsoft Office Excel. The relative quantification of mRNA levels was achieved by normalization of the mean of the gene expression of the gene of interest against the mean of the expression of the endogenous genes and resorting to the adapted equation $2^{-\Delta CT}$*1000. Results were analyzed using GraphPad Prism (version 6).

2.11. Exploratory literature review to find PROMs and ObsROMs

To find tools that can potentially assess PMM2-CDG patients' and caregivers' QoL, we searched Pubmed with pre-defined search terms until 19th April 2017. Two groups of search terms (Appendix 2) were employed: 1) QoL related and 2) PMM2-CDG related terms (including rare metabolic diseases terms and PMM2-CDG specific signs and symptoms as described on OMIM, MIM:212065). Search terms were deliberately broad as there is a huge diversity of PMM2-CDG clinical manifestations. Every combination of search terms from both groups were used connected by the Boolean operator "AND", i.e. "QoL term" AND "PMM2-CDG symptom". Resulting articles from our search were exported to Mendeley Desktop and duplicates were removed. Additional search for studies concerning parents' QoL was performed (Appendix 2). Also, references of relevant articles were screened and articles were included by author referral. To find eligible studies, the selection process was conducted in three major steps by two authors simultaneously. First, to obtain an adequate number of articles to analyze, an informatic selection was conducted resorting to a manufacturer's software and Structured Query Language (SQL), being the latter a domain-specific language designed for managing data held in a relational database management system (RDBMS), as Mendeley database. Several queries were built with different combinations of the search terms and executed to find the most suitable one for our study (Appendix 3). Selected studies had to contain in the abtract at least one term related with QoL, another related with rare inherited diseases and yet another PMM2-CDG related. Also, the articles had to be written in English and had to report at least one PROM or ObsROM. Reviews were excluded. In the second round of selection, all titles and abstracts were examined. Studies that could not be excluded in this examination entered the last phase of selection based on the analysis of the full-text version of each article. Data were extracted from the selected articles and any disagreements were arbitrated through discussion.

3. Results and Discussion

3.1. General introduction

Glycosylation is known to be crucial in many biological processes and altered glycosylation is related with many diseases influencing phenotypic maniphestations.[37]

Previous observations from our group suggested that sialidase treatment of human monocyte-derived DCs (moDCs) increase the expression of maturation markers, their genetic expression of pro-inflammatory cytokines and the priming and activation of T lymphocytes, highlighting the immunomodulatory role of Sia. This phenotypic alteration was attributed to the removal of α2,6 Sia, as shown by the analysis of ST6Gal-I$^{-/-}$ mice DCs.[137] The α2,6 sialylation is derived from the activity of different families of STs, specifically the ST6Gal and the ST6GalNAc families that differ in terms of their Sias acceptor specificity, galactose and GalNAc, respectively.[59] Besides its membrane bounded form, ST6Gal-I is also found in a biologically functional secreted form, which points towards an alternative pathway of extrinsic glycosylation that is able to modulate the cell surface glycosylation.[75,76,80,84,85] Therefore, we hypothesized that CRC cells secret ST6Gal-I that can modify the extrinsic glycosylation of immune cells and modulate their immune function.

CDG patients have immunological affectation, with the severity of immunological dysregulation being dependent on the CDG type.[118] In fact, glycosylation is involved in the majority of immunological processes including cellular recognition, signaling, lymphocyte development and homing.[40,42–47] Thus, it is expected that the defective glycosylation observed in CDG also influences the immune cells function. PMM2-CDG patients clinical manifestations include minor immunological involvement, such as recurrent and severe infections during childhood and fever-triggered stroke-like episodes.[124] Besides the wide clinical spectrum of PMM2-CDG patients, only management therapies are available for this CDG type. Therefore, unraveling the immune response affected mechanisms in PMM2-CDG compared to healthy individuals will pave the way into find curative treatment.

The lack of treatment, care guidelines and the heterogeneity of PMM2-CDG have a profound negative impact in patients and caregivers' QoL. The increased efforts to find therapy and cure for CDG are now highlighting the necessity of specific patient reported outcomes assessment tools for QoL evaluation as a primary endpoint in clinical trials. Thus, to find, adapt and/or validate suitable PROMs or ObsROMs would fulfill the existing gap and facilitate clinical outcome assessments.

The aims of this thesis comprised the assessment of the immunological aspects of glycosylation, specifically regarding CRC and CDG. Therefore, this thesis was divided in three parts: we intended (1) to study the aberrant glycosylation of CRC cells and its modulation in the immunological system, concerning CRC expression and secretion of ST6Gal-I and if this enzyme was able to extrinsically sialylate DCs affecting its phenotype and immune function; (2) to create a PMM2-CDG cell line to provide a new line of research to unravel immunological aspects of PMM2-CDG patients and evaluate their immune response to mitogenic stimulation, namely PMA and Ion and (3) to review the existing PROMs and ObsROMs to assess CDG patients' QoL and caregivers' QoL.

3.2. Part 1 – CRC and ST6Gal-I

3.2.1. ST6Gal-I expression analysis of CRC cell lines.

For the assessment of the first aim of this thesis, SW48 NC, SW48 ST6, SW948 NC and SW948 ST6 cell lines were initially cultured as previously described in the point 2.1.2. The original SW48 and SW948 cells were established in 1976 from a 82 year old female with Dukes' type C, grade IV colorectal adenocarcinoma and a 81 year female with Dukes' type C, grade III colorectal adenocarcinoma, respectively.[108] Both cell lines have nearly undetectable levels of ST activity.[141] Accordingly, the SW948 was shown to express low levels of α2,6 syalilation.[109] However, the activity in SW948 increased in a xenograph derived cell line showing that activity of this enzyme varies according to the requirements of growing conditions.[141] Unpublished data obtained from our group showed that transduction of the original cell lines with human cDNA of ST6Gal-I increased the content in α2,6 sialylation in the cell surface in contrast with the mock transfected (Figure 3.1), which is in concordance with previous studies resorting to dot blot SNA reactivity.[139]

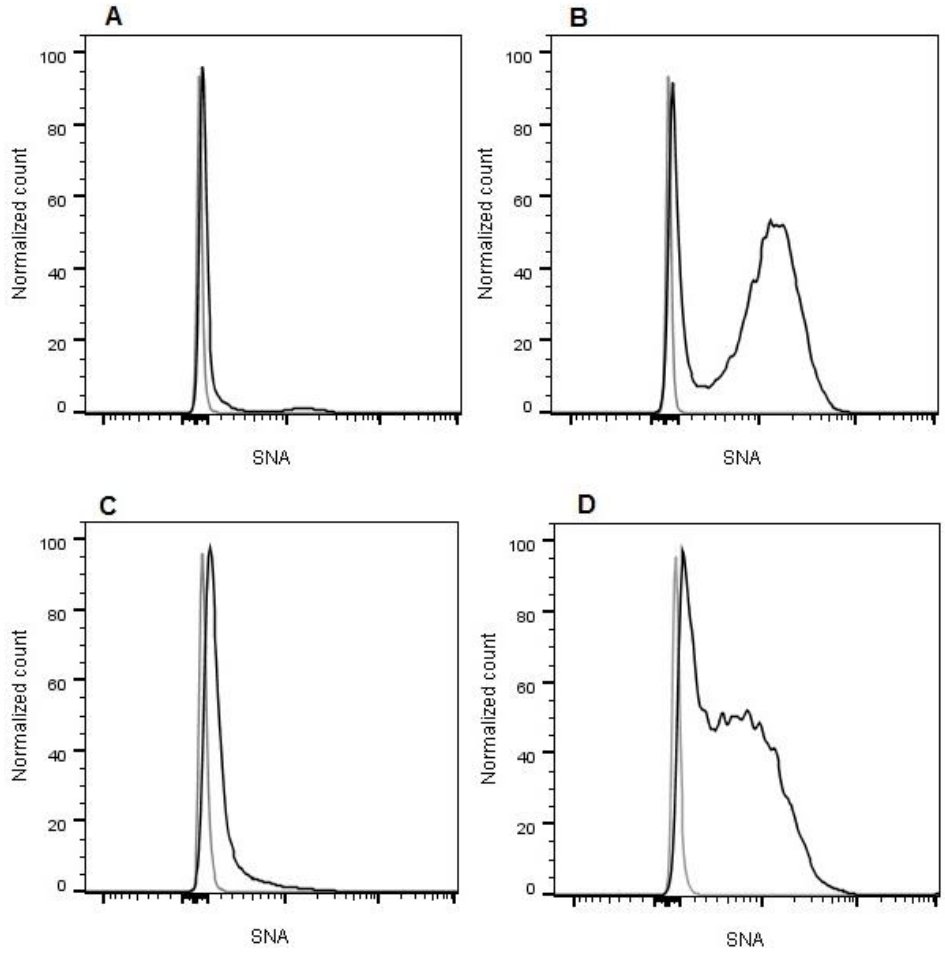

Figure 3.1 - α-2,6 sialylation of CRC cell lines. Flow cytometric analysis of the SNA staining of (A) SW48 NC, (B) SW48 ST6, (C) SW948 NC and (D) SW948 ST6 cell lines. In all 4 histograms, the *xx* axis represents the fluorescence intensity of the SNA-conjugated fluorophore and the *yy* axis represents the normalized event count. The grey line corresponds to the negative control (US cells) and the black line corresponds to the stained cells.

To check for the presence of the enzyme itself, WB was performed. WB is an analytical technique used to identify specific proteins from a cell homogenate or a complex mixture of substances. It involves the separation of proteins by gel electrophoresis and, then, the transfer of the separated proteins to a membrane where they can be detected using specific antibodies. The gel is composed by two different types where the pore size is modulated by the acrylamide concentration. In the upper side, there is the stacking gel with large pores to concentrate the samples in a thin starting zone, followed by the resolving gel with small pores where the proteins are separated.[142] In order to separate the proteins based only in their molecular weight, SDS-PAGE was used. SDS is an anionic denaturant agent that binds to the complex proteins, disrupting their secondary, tertiary or quaternary structure and confers them a negative charge. When an electric field is applied, the negatively charged proteins chains migrate through the gel towards the positive electrode, being the distance traveled dependent on their molecular weight.[143] Then, the proteins are transferred, the nonspecific sites of the membrane are blocked and the membrane is incubated with an Ab specific for the protein of interest (primary Ab). The unbound Ab is washed off and the membrane is incubated with a secondary Ab that detects the primary one. This secondary Ab is usually linked to a reporter enzyme such as alkaline phosphatase or HRP conjugates that cleave a chemiluminescent substrate that produces a detectable signal proportional to the amount of target protein. To assess the presence of ST6Gal-I enzyme, a homemade Ab was used and its specificity was tested with the purified enzyme and with WT and SG mice liver tissue lysates.

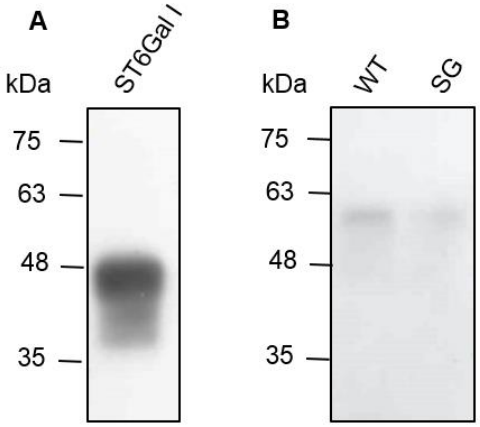

Figure 3.2 - Analysis of the anti-ST6Gal-I Ab specificity by WB. Assessment of the binding of the anti-ST6Gal-I Ab using (A) the purified enzyme ST6Gal-I and (B) WT and SG mice tissue lysates.

It was confirmed that the Ab recognizes the ST6Gal-I based on the appearance of a band approximately at 48 kDa (Figura 3.2A). It can be noticed that this band appears diffuse through a wide range of molecular weights probably because the enzyme has suffered some degradation while stored. Likewise, comparing the results of the WT and SG mice, there is a notable difference between the intensity of the ST6Gal-I bands, whereas the SG mice shows lower intensity (Figure 3.2B). Taking

into consideration that the knockout mice should not be able to express any protein, no band should be visible in the SG lane. In fact, in the generation of the SG mice, it was confirmed that the deleted exon produced a null mutation, i.e. a mutation that results in the complete failure of protein synthesis.[69] This exon encodes over 50% of the ST6Gal-I coding sequence and the N-terminal 200 aminoacids that codify the cytoplasmic and transmembrane catalytic regions. Therefore, even if any stable, truncated and active protein was expressed it could not enter or integrate the ER and Golgi compartment membranes as well as it would have a lower molecular weight.[69] The residual expression of ST6Gal-I may be due to the genetic background effects derived from genetic material of the embryonic stem cells on the ablated gene flanking regions, a common limitation while performing gene knockout.[144] It is also possible that the SG sample was contaminated with WT sample while loading them.

As the specificity of the Ab was proved, we checked if the enzyme was being expressed at the protein level by the CRC cell lines. In that way, a WB probing for ST6Gal-I on CRC cell lines lysates was performed.

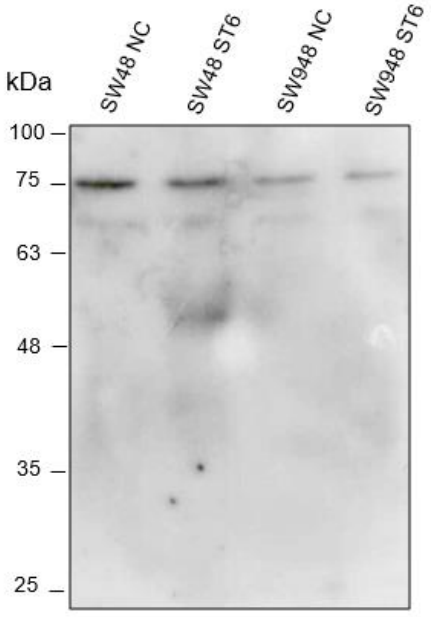

Figure 3.3 –ST6Gal-I expression at the protein level by CRC cell lines. Assessment by WB of the presence of ST6Gal I protein in the cell lysates of the SW48 NC, SW48 ST6, SW948 NC and SW948 ST6 cell lines using the anti-ST6Gal-I Ab.

SW48 ST6 was found to express ST6Gal-I in contrast to the other cell lines, as we can see by the appearance of a band around ~50kDa (Figura 3.3). This molecular weight (MW) is in concordance with previous reports in colorectal, ovarian and pancreatic cancer.[138,145] The variation on the MW could be due to differences in the protein glycosylation. In fact, ST6Gal-I has two N-glycosylation sites in the residues Asp 149 and Asp 161.[64] Unexpectedly, SW948 ST6 was not found to express ST6Gal-I, although transduced. The undetectable ST6Gal-I protein expression in this cell

line, could be due to the fact that all the enzyme was being secreted to the extracellular medium (assessed in the topic 3.2.2). On the top of the gel we can notice bands of higher MW (~75kDa and ~70kDa) that consist of unspecific binding of the primary or secondary Abs to the membrane. To try to reduce the unspecific binding, the Ab concentration should be minimized and/or the Ab solutions should be diluted in TBST-0.1% supplemented with non-fat milk powder.[146]

3.2.2. Assessment of ST6Gal-I secretion by CRC cell lines.

Since we found out that SW48 ST6 cell line expressed ST6Gal-I, next we assessed if the enzyme could secrete the enzyme to the extracellular medium. For that, cells were cultivated in DMEM with 2% FBS and supernatants were recovered at different time points and analyzed by WB.

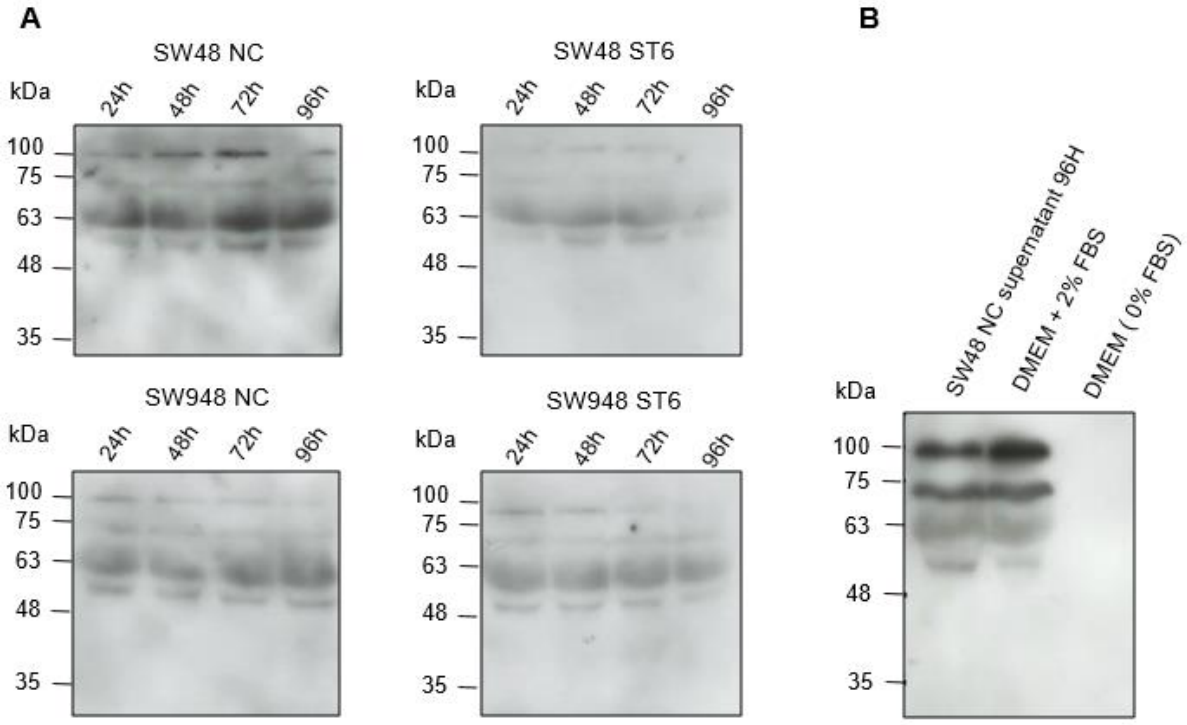

Figure 3.4 –ST6Gal-I secretion to the extracellular medium by CRC cell lines. Analysis by WB of the presence of the ST6Gal-I enzyme using the anti-ST6Gal-I Ab in (A) the supernatants of the SW48 NC, SW48 ST6, SW948 NC and SW948 ST6 cell lines at 24 H, 48 H, 72 H and 96 H and (B) the medium DMEM with 2% FBS or without FBS compared with the 96 H supernatant of the SW48 NC cell line (control).

The revelation of the membrane has shown a similar band pattern of unspecific bands among each one of the cell lines (Figura 3.4). In fact, when probing a membrane with samples of medium with and without FBS, the same band pattern lighted up in the lane with DMEM supplemented with 2% FBS in contrast with the lane without any FBS, which shows that the detected proteins were components of the FBS but not secreted products from the cells. Thus, the protocol suffered optimization and the cells were cultured in DMEM without serum as described in the topic 2.5.

However, after performing WB with the new supernatants, no bands were detected at any time of any cell line (data not shown). A possible explanation for the absence of any band could be that the secreted ST6Gal-I concentration was much lower compared with other secreted proteins and consequently, the quantity of ST6Gal-I loaded in the gel was too low to be detected. Therefore, the supernatants were concentrated with centrifugal filters with a molecular cutoff weight of 15000 kDa in order to discard every protein with a lower MW and consequently, enrich the concentration of ST6Gal-I in the supernatant.

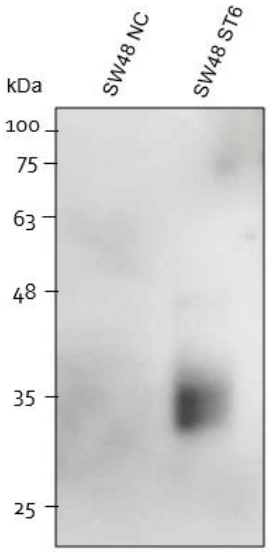

Figure 3.5 - ST6Gal-I secretion to the extracellular medium by the SW48 NC and SW48 ST6 cell lines. Analysis by WB of the presence of ST6Gal-I using the anti-ST6Gal-I Ab in the supernatants at 72 H. Supernatants were 12 times concentrated.

The results of the WB with the concentrated samples have shown that the SW48 ST6 cell line is capable to secrete the ST6Gal-I soluble form, as we can see a band of ~35 kDa (Figure 3.5). Similar to what was already mentioned regarding to intact ST6Gal-I, the MW found for the cleaved protein is in concordance with previous reports in colorectal, ovarian and pancreatic cancer.[138,145] In contrast with what was hypothesized before to explain the fact that no protein was being expressed inside the cell, SW948 ST6 was found to be incapable of secreting ST6Gal-I (data not shown). The lack of expression of the ST6Gal-I enzyme can be due to epigenetic inactivation of the ST6Gal-I gene, specifically by DNA hypermethylation or histones acetylation. Normal DNA methylation and histones acetylation patterns are essential for maintaining a correct gene expression. However, these patterns are often dysregulated in cancer.[147] In fact, ST6Gal-I gene silencing due to aberrant DNA methylation in the promoter region has been reported before in glioma and bladder cancer.[147,148] In this situation, CpG islands (segments of the DNA chain with atypically high frequency of CpG sites) in promoter regions are hypermethylated which blocks the binding of transcription factors to specific promoters and consequently causes gene silencing.[149] Moreover, DNA methylation enhances the binding of

methyl-binding proteins, including histone deacetylases. Histone deacetylation results in an increase in the positive charge of the DNA chain and in a subsequent compact chromatin structure inaccessible to the transcription machinery.[150] However, in the glioma context, histone deacetylation seems to play a minor role in the regulation of ST6Gal-I expression.[147]

3.3. Part 2 – CDG immunological assessment

3.3.1. PMM2-CDG-PBMCs Immortalization

In the second part of this project, it was hypothesized that the defective glycosylation observed in PMM2-CDG patients also modulates the function of immune cells. For that, the first goal was to create a PMM2-CDG PBMC-derived cell line, preferably a monocyte or lymphocytic cell line that would allow us to have a model to explore the immunological affectation on PMM2-CDG in this project and future ones.

Although primary cells provide cultures with very similar characteristics to the origin source, they have a limited life-span. The cell types that allow sub-culturing are very limited and a great variability exists between cell preparations. The introduction of immortalized cell lines in the cell biology research field allowed to overcome these limitations. In fact, they escape the fine control of cell cycle allowing the cells to continuously divide and become simpler models for biological systems[151]. One of the immortalization methods widely used is the lentiviral transduction of cells. Although some types of cells offer resistance to transduction, lentiviral vectors can transduce either proliferating or non-proliferating cells.[152] The EBV gene has been used to achieve cell immortalization as EBV infected cells express latent viral proteins and maintain their ability to grow due to deregulation of telomerase reverse transcriptase (TERT) activation, a key enzyme involved in cellular replication.[153] Although EBV is associated with infectious mononucleosis and malignancy, the enhanced proliferation is controlled by T_c cells that recognize the viral markers at infected cells surface, maintaining a latent infection.[154] As infection with EBV is well known to immortalize primary non-dividing lymphoblastic cells[155,156], we attempted to immortalize PBMCs populations in general resorting to lentiviral transduction of the EBV gene. The isolation of PBMCs was achieved resorting to Biocoll based on the fact that this solution is denser than PBMCs, but has a lower density than the remaining blood cells, allowing the formation of a PBMCs' layer easy to isolate. As for the immortalization process, hexadimethrine bromide (with the commercial name of polybrene) was used as it is a polycation that neutralizes charges interactions to increase binding between the pseudoviral capsid and the cellular membrane.[157] ViralPlus was used to increase lentiviral transduction efficiency, because it is a chemical cocktail that suppresses the cellular anti-viral state by mimicking the activity of viral virulent gene products *in vitro*.[158]

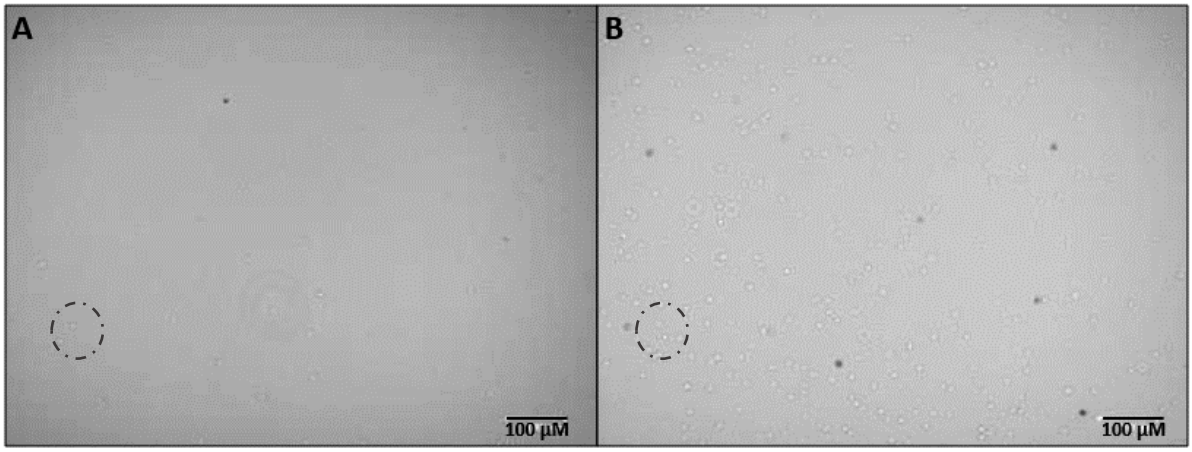

Figure 3.6 – Microscopy images of PMM2-CDG-PBMCs before and after the immortalization process. (A) PMM2-CDG-PBMCs at low cell density and (B) EBV-immortalized PMM2-CDG-PBMCs at high cell density. The black circunference evidenciates the difference between cell density in each situation.

After the immortalization process, cells started to proliferate as the cell density in immortalized cells (Figure 3.6B) increased compared to the original PMM2-CDG-PBMCs (Figure 3.6A). Furthermore, cell morphology of infected PMM2-CDG-PBMCs remained similar to its primary counterpart. The EBV gene expression was assessed by RT-qPCR to evaluate the success of the immortalization process. The results showed detectable expression of the EBV gene in the immortalized PMM2-CDG-PBMCs with a threshold cycle (C_T - the cycle number at which the amount of amplified target reaches a fixed threshold) value of 22.47 at passage 13, but not in the control, this is, the non-immortalized PMM2-CDG-PBMCs. However, at passage 15, the genetic expression of the transgene seemed to have reverted, as the RT-qPCR gave a C_T value of 34.97. Along with this decrease in the EBV expression, the cells appeared to have reached senescence as they aggregated and stopped proliferating (Figure 3.7).

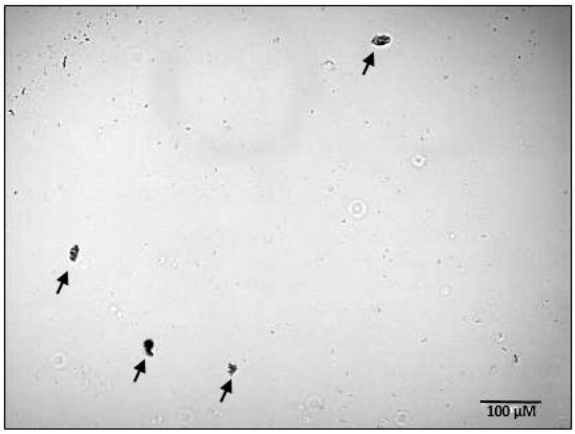

Figure 3.7 - Microscopy image of senescent PMM2-CDG-PBMCs. EBV-immortalized PMM2-CDG-PBMCs at the passage 15 after immortalization. Black arrows indicate cell aggregates.

The unsuccessful immortalization of PMM2-CDG-PBMCs could be due to the preferential lentiviral genome integration in active transcription sites which can cause the disruption of essential host genes.[159] However, the hyperproliferation of EBV-infected B cells has previously been observed to become growth arrested through the activation of the ataxia telangiectasia mutated Ser/Thr kinase (ATM)/checkpoint kinase 2 (Chk2)-dependent DNA damage responses (DDRs) *in vitro* that was reported to regulate the oncogene-induced senescence.[160,161] Moreover, enhanced proliferation gives rise to an increased need for metabolic intermediates that is counterbalanced by the Warburg effect, i.e. the up-regulation of autophagy and the activation of the alternative aerobic glycolysis metabolic profile (the conversion of glucose to lactate in the presence of oxygen).[162,163] Recently observations showed that the senescent B cells have a reduction in normal mitochondrial activity that restricts EBV-mediated B-cell proliferation, along with DDRs.[164] Therefore, both metabolic imbalance and DDRs can be the origin to the observed final senescent state in PMM2-CDG-PBMCs after the hyperproliferating period. In addition, previous observations suggested that EBV gene expression is not sufficient to fully immortalize EBV-infected B cells, but TERT expression is also necessary. In fact, although EBV-infected lymphoblastic cell lines (LCLs) with no TERT expression had a prolonged life-span, they lost proliferation at some point in contrast with fully immortalized LCLs cells with TERT expression.[165] Unfortunately, the expression of this enzyme was not analyzed in PMM2-CDG-PBMCs before or after the immortalization process and thus, we cannot infer that they were not expressing it. Either way, further attempts to perform immortalization should include co-transduction with EBV and TERT genes, as well as other immortalization agents.

3.3.2. Assessment of PBMCs populations

Before freezing the cells to stock them until further studies, PBMCs populations were evaluated for both PMM2-CDG patient and his healthy sibling. Results are summarized in Table 3.1.

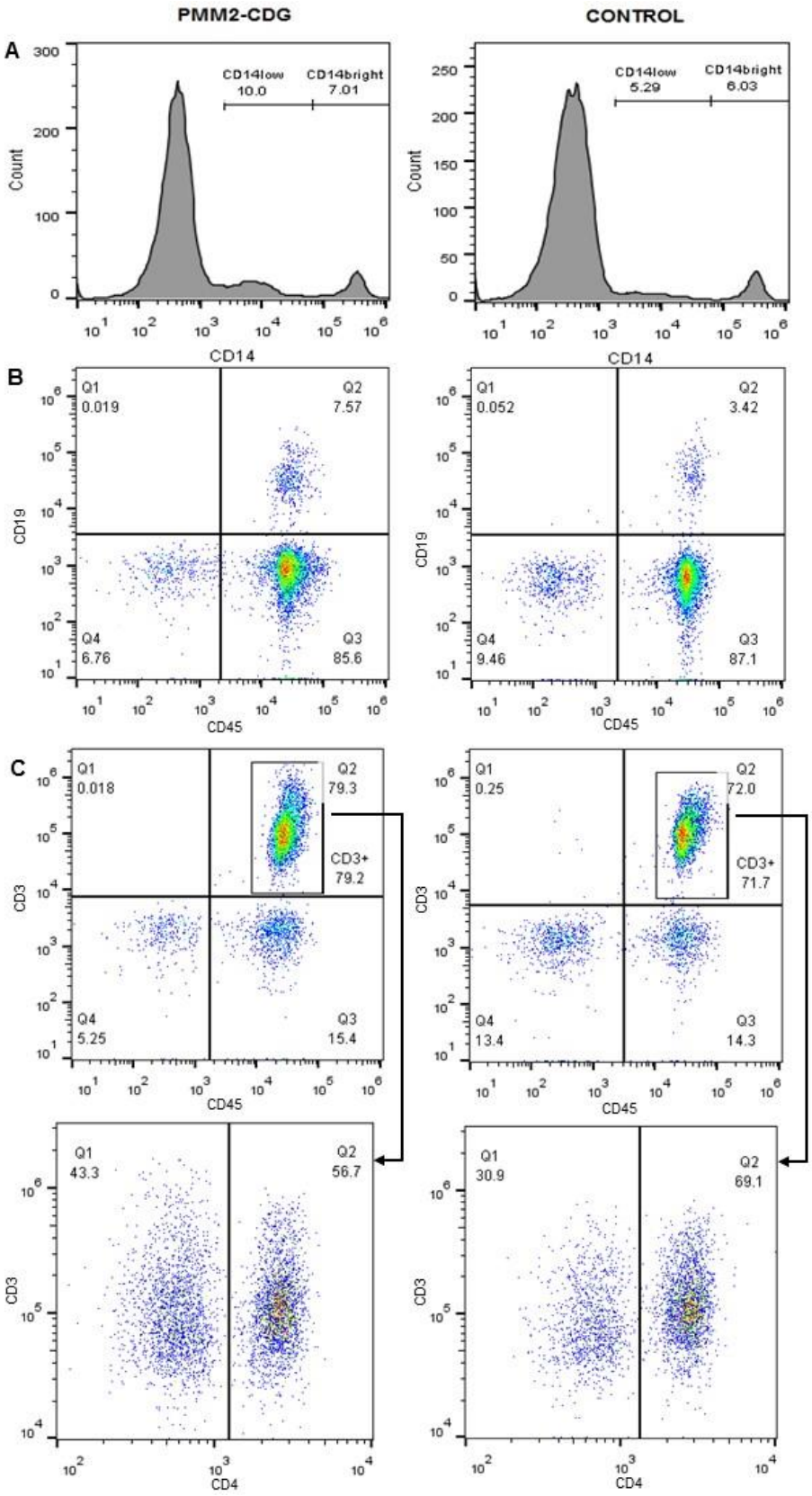

Figure 3.8 - Blood populations analysis by flow cytometry. Assessment of (A) monocytes, (B) B lymphocytes and (C) T_C and T_H lymphocytes populations of the PMM2-CDG donor (left) and his healthy sibling (right).

The CD14 marker was used to quantify the monocyte population (Figure 3.8A). Other cells express CD14, namely macrophages and granulocytes[166], however, macrophages are not circulating cells and granolocytes were excluded by immunomagnetic separation which allow us to specifically analyse the monocytes. The results indicate that the PMM2-CDG patient has an enriched population of monocytes in comparison to the healthy sibling, with 17.1% and 11.32% of monocytes, respectively (Tabela 3.1). The monocyte percentage of PMM2-CDG patient is significantly different from the reference value of ~4-10%, while the percentage of the control donor is not so different.[167] In the Figure 3.8A, two $CD14^{+}$ populations with differencial expression of this cell surface marker can be distinguished, here after called $CD14^{bright}$ and $CD14^{low}$, with higher and lower expression of CD14, respectively. The main difference between the monocyte subpopulations of PMM2-CDG patient and his sibling is the higher amount of $CD14^{low}$ population, with 10% in contrast with 5.29%, respectively. Monocytes are divided into three subsets based on the expression of two cell surface markers: the classical monocytes with high expression of CD14 and no expression of CD16; the intermediate subset with high expression of CD14 and low expression of CD16 and the non-classical subset with high expression of CD16 but lower expression of CD14.[168] While classical monocytes were reported to have phagocytic activity, the non-classical monocytes were suggested to have inflammatory properties upon activation based on the increase of this population during inflammation and pro-inflammatory cytokine production. The intermediate monocytes showed both inflammatory and phagocytic capabilities.[169] The $CD14^{low}$ populations observed in Figure 3.8A, can possible correspond to the non-classical monocyte subset, however, to sustain this hypothesis, the cells should have been stained for CD16 which implied a previous exclusion of NK cells that also express CD16. If the $CD14^{low}$ population indeed have inflammatory properties, there might be two explanations: (1) the blood take was done in a timepoint in which the PMM2-CDG patient was subjected to a pathogen despoleting an inflammatory response or (2) the PMM2-CDG can be in a permanent inflammatory state due to the response to accumulated underglycosylated proteins inside the ER, as previously suggested by Heyne *et al.* 1998.[129]

The B cells population was assessed based on the expression of the CD45 and CD19 markers. While the healthy sibling B cells corresponded to 3.42% of the PBMCs population, PMM2-CDG B cells had a percentage of 7.57% (Figure 3.8B). Although PMM2-CDG patient B cells were found to be doubled, this value is still inside the reference value of ~2-10% (Table 3.1).[167] Regarding T cells, evaluated based on CD45, CD3 and CD4 (for T_H cells) and CD8 (for T_C cells) markers, the PMM2-CDG patient presented higher levels of T_C cells and lower levels of T_H cells, with percentages regarding $CD3^{+}$ cells of 43.3% and 56.7%, respectively, contrasting with the healthy sibling with 30.9% and 69.1%, respectively (Figure 3.8C). While the control donor percentages were similar to the reference values (~30% and ~70% of T_H and T_c cells, respectively[167]), the percentages of PMM2-CDG lymphocytes were found altered (Table 3.1). The increased percentage of B cells and T_C cells, the lower levels of T_H cells along with the high monocyte PMM2-CDG populations mentioned before

are consistent with a state of infection, whereas lymphocytes populations would expand in response to the pathogenic agent.

Table 3.1 - Summary of PBMCs populations percentages of the PMM2-CDG patient and the healthy sibling. Reference values accordingly to Invitrogen cell count table.[167] *percentage regarding $CD3^+$ population.

		PMM2-CDG	CONTROL	Reference Value
Monocytes	Total	17.10 %	11.32 %	~4-10%
	CD14low	10.00 %	5.29 %	-
	CD14bright	7.01 %	6.03 %	-
B cells	Total	7.57 %	3.42 %	~2-10%
T cells	Cytotoxic	43.30 %*	30.90 %*	~30%
	Helper	56.70 %*	69.10 %*	~70%

3.3.3. Optimization of the T cell mitogenic stimulation and proliferation

We aimed to test the differences in the mitogenic immune response between the PMM2-CDG patient and his healthy sibling. Since the immortalization was unsuccessful, these studies were done with the frozen PBMCs. However, the protocol to perform both cell staining and stimulation had to be optimized because there was a limited stock of frozen cells from both donors and, after defrosting the cells, it was expected that the cells had lost viability.

To choose the best condition for stimulation, CFSE was used at a concentration previously optimized by our group and cells were subjected to different concentrations of PMA and Ion. CFSE Cell Proliferation Kit (as well as the others CellTrace™ Kits) work on the basis that the dye with succinimidyl esters cross the cellular membrane and after the action of intracellular esterases, bind covalently to primary amines located inside the cells, being this fluorescence stable and well-retained (Figure 3.9). When cells divide, the fluorescent probes also divide, being the fluorescence intensity of the daughter cells half of the intensity of the mother cells. Therefore, it provides a way to follow multiple generations after cell stimulation.[170]

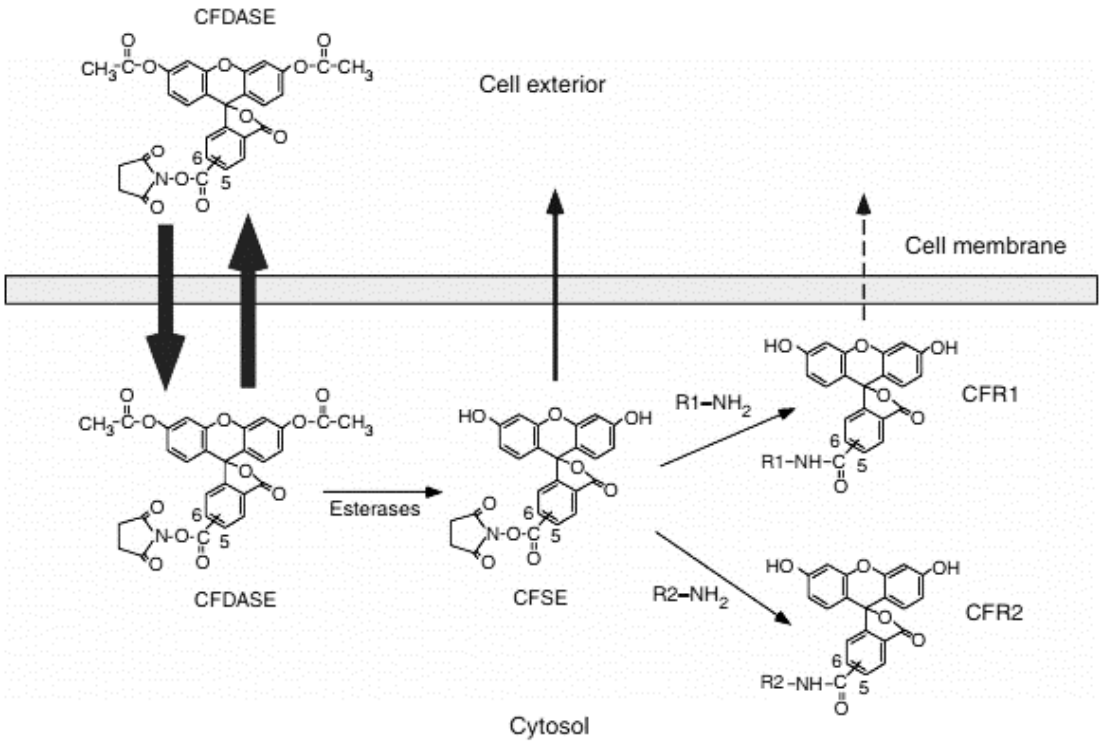

Figure 3.9 – Mechanism of cell staining using CFSE. Once the CFSE non-fluorescent precursor CFDASE enters the cells, the acetate group is removed by intracellular esterases, exposing the succinimidyl group of the fluorescent CFSE. CFSE stays inside the cell due to its low cell permeability where it reacts to amino groups of proteins to form CFR1 or CFR2 conjugates. CFR1 represents conjugates that have the capacity to exit the cell or are rapidly degraded and CFR2 are the conjugates that become stable inside the cells. From Quah *et al*. 2007.[171]

Moreover, the optimization of the stimulation process was done not only to PBMCs (Figure 3.10) but also to $CD3^+$ cells obtained by immunomagnetic separation (Figure 3.11). This method has been increasingly used to isolate a specific population from a mixed cell population and involve the attachment of small magnetically active particles (beads) to specific markers of the cells of interest. In this case, Ab-coated beads against CD3 were used which allowed to apply a magnetic field that immobilized the labeled $CD3^+$ cells into the column while the negative fraction was eluted – MACS separation.[172]

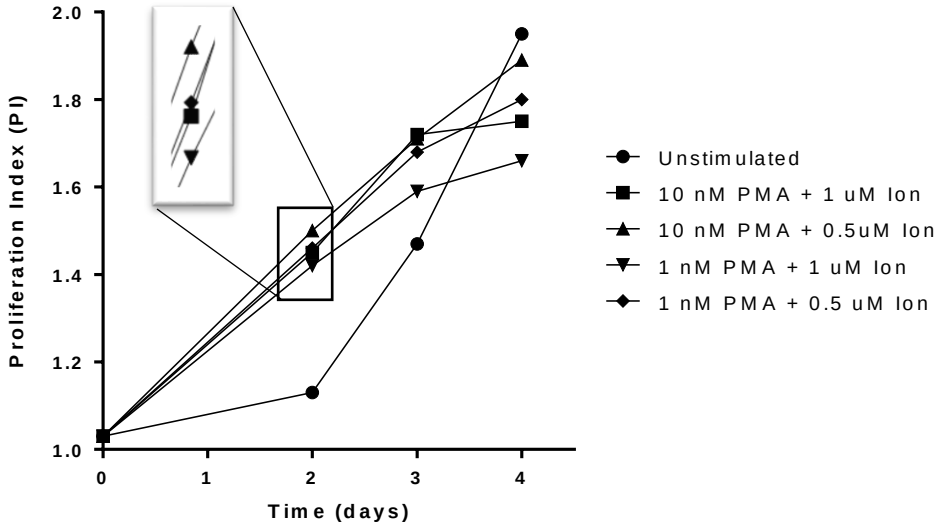

Figure 3.10 – Optimization of T cell mitogenic stimulation by flow cytometry. CFSE-stained PBMCs were stimulated with 4 different combinations of PMA and Ion and the proliferation index (PI) were obtained over time.

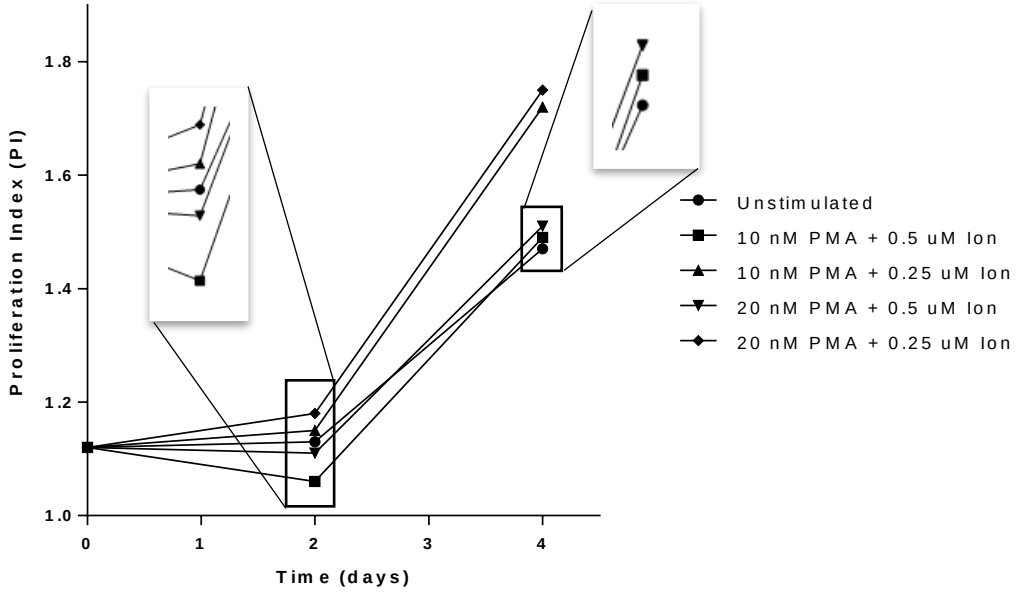

Figure 3.11 – Optimization of T cell mitogenic stimulation by flow cytometry. CFSE-stained $CD3^+$ cells were stimulated with 4 different cmbinations of PMA and Ion and the proliferation index (PI) was obtained over time.

In Figure 3.10, we can observe that the unstimulated PBMCs started to proliferate which was not expected. Moreover, unstimulated cells ended up proliferating more than the stimulated PBMCs. This event can be explained by a possible external contamination of the medium.

From the mitogenic stimulation of PBMCs (Figure 3.10), we can infer that using a higher concentration of PMA (10 nM) results in a higher proliferation index than using a lower concentration (1 nM). However, we tried an even higher concentration of PMA (20 nM) when stimulating $CD3^+$ cells, but the result turned to be similar to the stimulation with 10 nM of PMA (Figure 3.11), which may indicate that, after a certain threshold, the activation of PKC by the mitogen becomes saturated, accordingly with previous studies.[173]

In Figure 3.10, we can observe that despite differential concentration of PMA, using 0.5 µM instead of 1 µM of Ion to stimulate PBMCs results in higher proliferation indexes. In the same manner, in Figure 3.11, 0.25 µM of Ion worked better than 0.5 µM in terms of $CD3^+$ cell proliferation. Therefore, we can infer that using a lower concentration of Ion provides better results for proliferation. IL-2 was added to the culture medium to provide T cells a signal of survival and proliferation. Physiologically, IL-2 is mainly produced by T_H cells after antigen recognition and binds to autologous or allogeneic IL-2 receptor (IL-2R) to control T cell function survival, proliferation and differenciation.[1] Therefore, the lower proliferation capacity observed with higher concentrations of Ion can be due to a concentration-dependent inhibition of IL-2R expression in the T cells surface, as previously reported by Chopra *et al.* 1987.[21] In fact, they reported that conjugation of PMA with lower concentration of Ion was highly synergistic, whereas conjugation with high Ion concentration was inhibitory in terms of IL-2R mRNA accumulation, IL-2R expression and proliferation.[21]

When purified $CD3^+$ T cells are stimulated with PMA plus Ion there is a resistance in proliferation, as we can see that they only proliferate in a higher extent after 48 H (Figure 3.11). This result can be explained by the absence of other cell populations required for T cell activation. Previous observations have shown that monocytes indeed are required for T cell activation in response to mitogens, playing an active role in IL-2 secretion, IL-2R expression and cell proliferation.[26] However, the need for accessory cells is controversial, but as pointed out by Alter and Bach 1970, differential results are well explained by the differential degrees of contamination with other cells of the supposedly purified lymphocytes.[174] Finally, the addition of exogenous IL-2 to the medium was previous found to restore the levels of T cells responsiveness, which contrasts with our observations.[26]

Taking into consideration the observations above, we proceeded with studies to find the optimal PMA plus Ion stimulus to challenge PBMCs mixed populations.

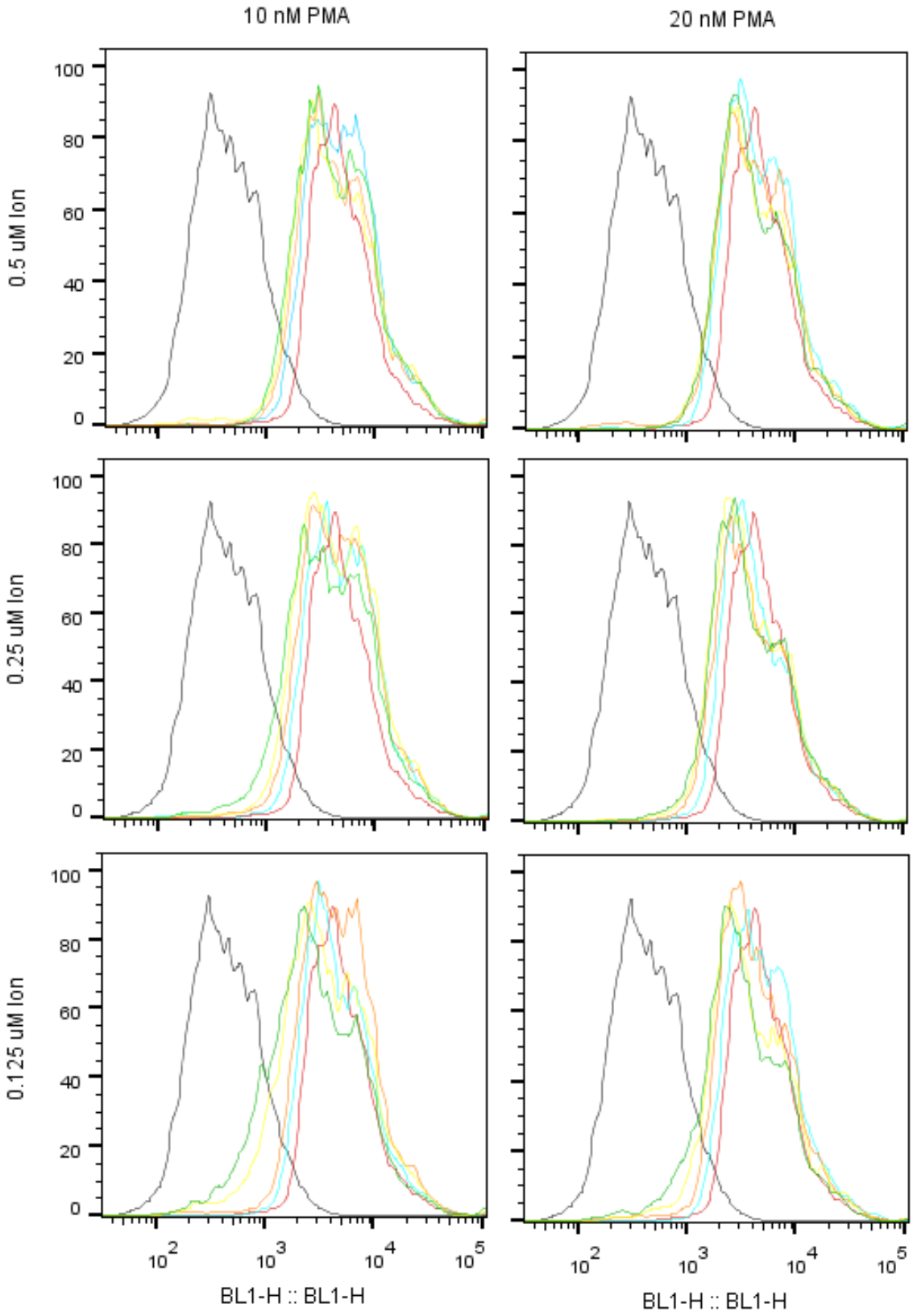

Figure 3.12 - PBMCs stimulation optimization by flow cytometry. CFSE-stained PBMCs subjected to 6 combinations of PMA and Ion during 24 H (blue), 48 H (orange), 72 H (yellow) and 96 H (green), unstimulated CFSE-stained PBMCs (red) and US cells (black).

By the analysis of Figure 3.12, we can see that, with both PMA concentrations used, the lower is the Ion concentration of the stimulus (from top to the bottom), the higher is the shift in the CFSE staining, which means a higher extent of cell proliferation. However, the difference between different concentrations of PMA with the same concentration of Ion is not visible without further analysis. Therefore, the proliferation indexes over time were calculated for each one of the 6 stimuli.

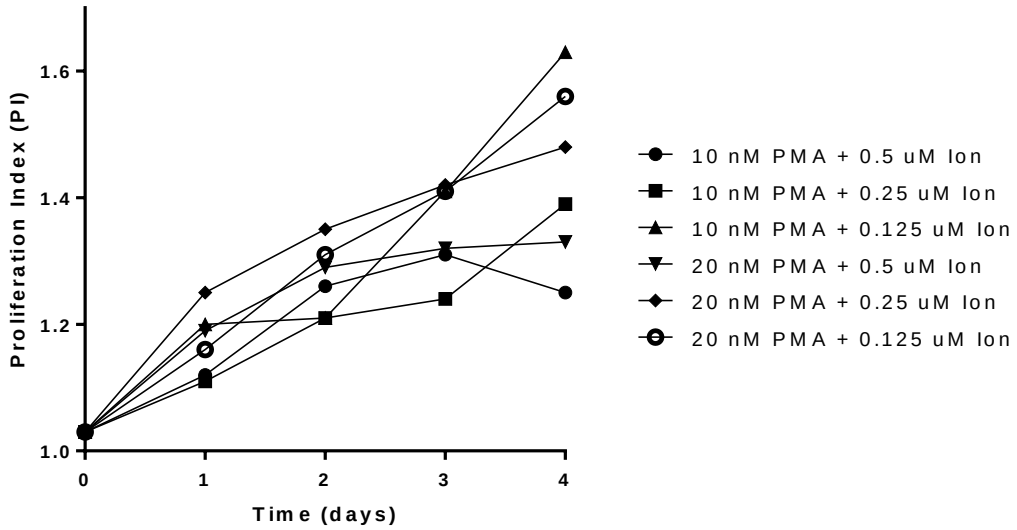

Figure 3.13 - PBMCs mitogenic stimulation optimization by flow cytometry. PBMCs' PI over time using 6 different combinations of PMA and Ion .

The results represented in Figure 3.13, corroborate our previous observations (Figures 3.10 and 3.11), as we can see that higher Ion concentrations are responsible for lower final proliferation indexes, and vice-versa. About the PMA concentration, it can be inferred that a higher concentration causes a higher T cell proliferation in an early point of stimulation (day 1), in contrast with lower concentrations of PMA, which activate T cells in a slow and increasing manner, with higher proliferation rates in a later stage (day 4). As the 10 nM PMA plus 0.125 µM Ion was the stimulus that resulted in a higher proliferation index, this stimulus was chosen to challenge the PMM2-CDG patient and his healthy sibling PBMCs.

Besides using CFSE, that has been shown to decrease proliferative capacity due to a decrease in the proliferating cell viability in a concentration-dependent manner,[175] it was also attempted to stain the cells with CellTrace™ Far Red in order to use the less aggressive dye when stimulating the PMM2-CDG and control cells.

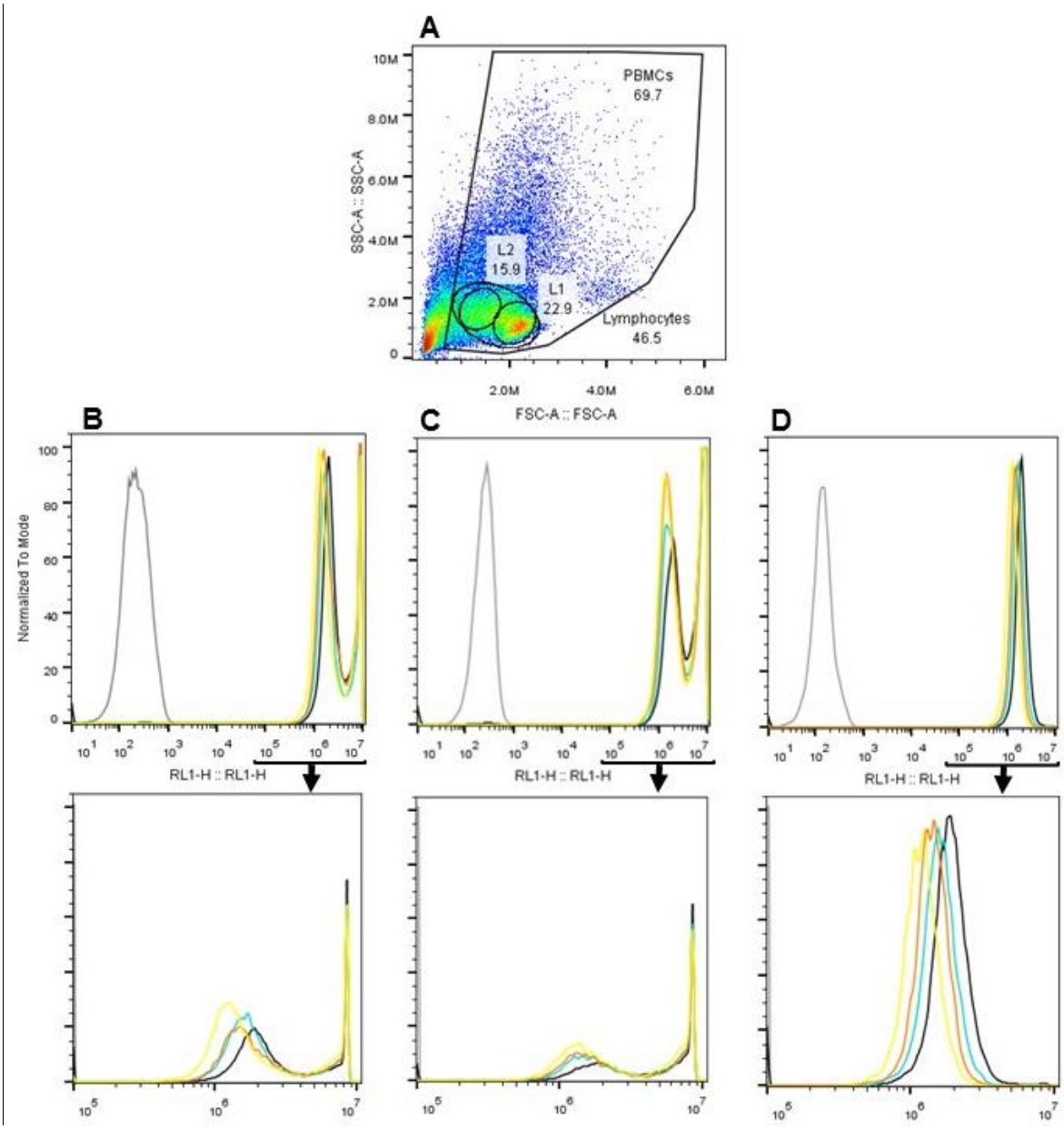

Figure 3.14 – Optimization of the PBMCs CellTrace™ Far Red staining by flow cytometry. Three different gating strategies were employed, namely, PBMCs, and two different lymphocytic populations, L1 and L2 (A). CellTrace Far Red-stained cells subjected to 10 nM PMA plus 0.125 µM during 24H (blue), 48H (orange) and 72H (yellow), CFSE-stained PBMCs without stimulation (red) and unstained cells (black), gating PBMCs (B), L2 (C) or L1 (D).

The staining with 1 µM of CellTrace™ FarRed resulted in a very bright staining, as we can see by the position of the peaks in the limit of the axis, so the dye concentration could be reduced (Figure 3.14B, C and D). It is also observable the existance of two different peaks in the PBMCs stained cells (Figure 3.14B). Both peaks are present in the unstimulated stained cells, so the lower intensity (LI) peak is not derived from a decrease in fluorescence intensity due to proliferation, but instead cells of first generation are in the origin of both peaks. Moreover, the higher intensity (HI)

peak is not derived from the existence of doublets or clumps as the gating strategy applied excluded them at first. Gating subpopulations (Figure 3.14A), we can see that the LI peak corresponds to the subpopulation L1 (Figure 3.14C), being the HI peak derived from the other PBMCs populations, including a lymphocytic subpopulation, here called L2 (Figures 3.14A and 3.14B). Moreover, we can see a shift in the LI peak and not in the HI one meaning that L1 subpopulation is the one that proliferates. Therefore, it is likely that L1 population correspondes to the activated T cells as it has as higher forward scatter (FSC) (Figure 3.14A). In fact, activation of T lymphocytes is associated with an increase in the FSC signal.[176] Previous assays preformed by our group show that the staining of fresh PBMCs originate one peak only (data not shown). Therefore, the appearance of the two peaks is probably due to the fact that this assay was done with frozen cells and so they probably have lost integrity at some extent.

To analyse if the dyes staining was affeting the cell functioning, the genetic expression of a cytokine, TNF-α was measured by RT-qPCR and quantified by the method introduced in 2001 by Livak and Schmittgen.[140] This method describes the variation in the expression of a certain gene in comparison to a control (for instance, an untreated sample). While the C_T indicates the cycle number at which the amount of amplified target reaches a fixed threshold, ΔC_T stands for the difference between the C_T of the target gene and that of the endogenous controls. This intrinsic controls must be constitutively expressed, i.e., with a stable expression regardless any experimental conditions. This step of normalization provides a way to correct results for different amounts of input RNA.[140] Here, it was used an adaptation of the $2^{-\Delta\Delta CT}$ method that infers about the number of messenger RNA (mRNA) molecules of the gene of interest per 1000 of molecules of the endogenous gene, given by the equation $2^{-\Delta CT*1000}$.[177]

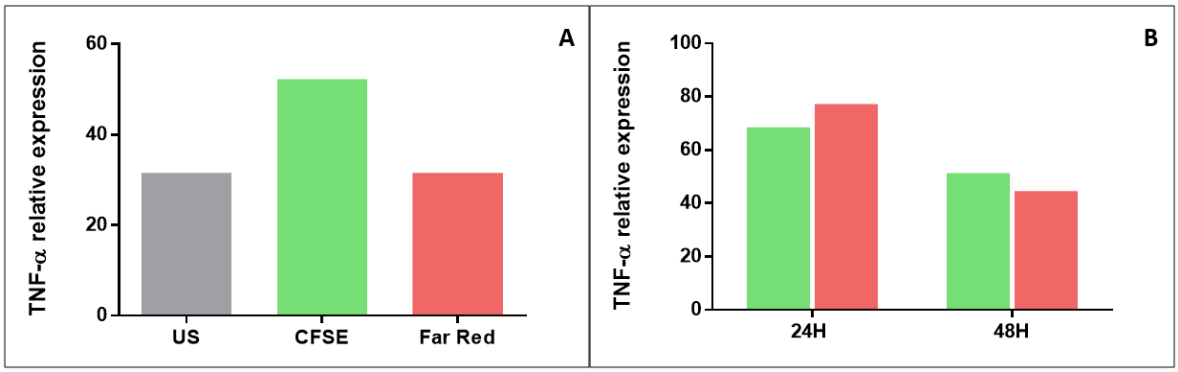

Figure 3.15 – TNF-α genetic expression analysis by RT-qPCR. TNF-α relative expression of (A) (grey) unstained PBMCs, (green) CFSE stained PBMCs and (red) Far Red stained PBMCs 18 hours after staining and (B) CFSE (green) or Far Red (red) stained PBMCs 24 and 48 hours after PMA and Ion stimulation.

The measure of the expression of the TNF-α showed that CFSE stained cells have an altered expression compared to the unstained cells without any stimulus, in contrast to CellTrace™ Far Red

stained cells (Figure 3.15A). This result show that PBMCs staining with CFSE is influencing the cell functioning *per se*, without any addicional procedure. The increased expression of TNF-α when staining with CFSE can be due to the release of DAMPs in response to CFSE-derived cell damage/stress. For instance, heat-shock proteins (HSPs) are released from cells under stress and initiate innate immune responses, including the production of TNF-α by macrophages.[178] Therefore, this result suggests that CFSE is cytotoxic to the defrosted PBMCs which causes the macrophages to become activated and express TNF-α to eliminate the harmed cells. However, the concentration of CFSE in the staining protocol could not be decreased because the stained cells peak would overlap with the unstained cells, affecting the measures of the proliferation capacity. However, the relative quantification of the TNF-α expression 24 and 48 H after stimulus shows a similar pattern independently of the proliferation dye used, i.e. the expression of TNF-α is higher 24 H after stimulus and decreases after 48 H. Considering the previous results, we decided to proceed with stimulation of PMM2-CDG and his healthy sibling cells using CellTrace™ Far Red as proliferation dye because stained cells behaved similarly to unstained cells without stimulus.

3.3.4. Evaluation of the PMM2-CDG mitogenic response

After the optimization of the staining with the proliferation dye and the mitogenic stimulation, we proceeded with the ultimate goal of test the differences in the mitogenic immune response between the PMM2-CDG patients and the corresponding healthy sibling, i.e. control with the same genetic backgroud but without any mutation in the *PMM2* gene, namely in terms of cell proliferation and cytokine expression and production. Cells were stained with CellTrace™ Far Red and stimulated with PMA and Ion.

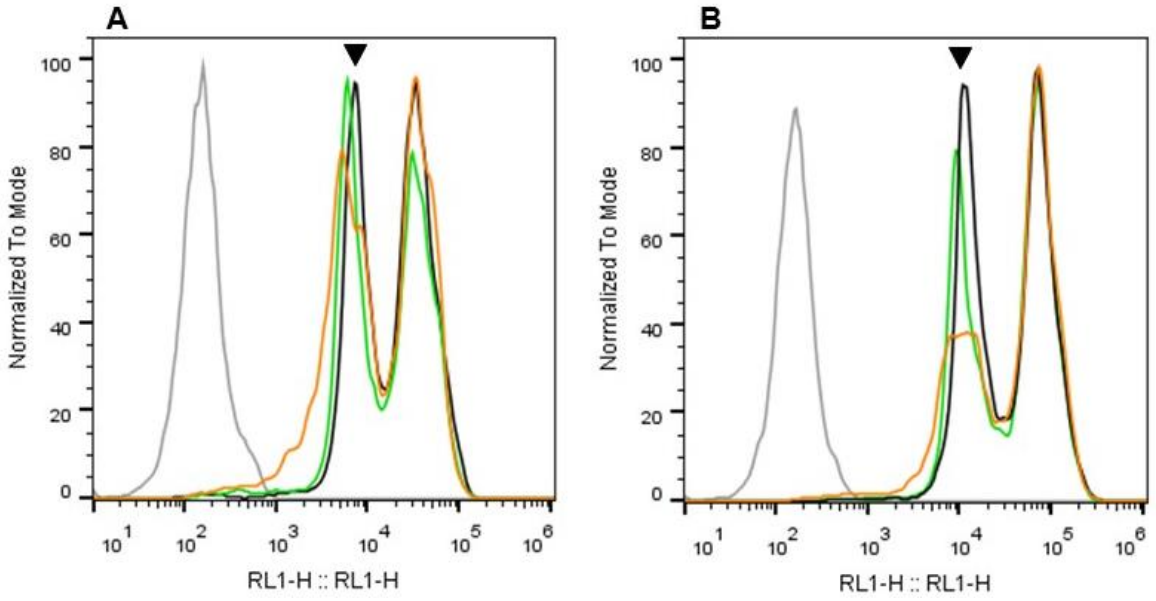

Figure 3.16 – PMM2-CDG cells proliferation capacity analysis by flow cytometry. 0,05 µM CellTrace™ Far Red-stained PBMCs subjected to 10 nM PMA plus 0.125 µM during 24 H (green) and 72 H (orange),0,05 µM CellTrace™ Far Red-

stained PBMCs without stimulation (black) and unstained cells (grey), gating overall PBMCs of (A) PMM2-CDG patient and (B) the healthy sibling. ▼ indicates the MFI of the LI peak.

As we can see in Figure 3.16, the staining of both PMM2-CDG-PBMCs and the control PBMCs originate also two peaks with different intensity, LI peak and HI peak. Moreover, just the LI peak suffers an intensity shift derived from proliferation, which is in concordance with our previous observations when optimizing our protocol. Moreover, PMM2-CDG cells have a bigger decrease in intensity at 72 H after stimulation in contrast with control cells, which suggests that PMM2-CDG patient's T cells have a higher proliferation capacity than the corresponding healthy sibling's T cells. To corroborate this observation, we also analyzed the PIs, considering the median fluorescence intensity of the LI peak as the minimal intensity of the first-generation cells. Therefore, every cell that presented less intensity that this LI peak after stimulation would be considered as proliferation products.

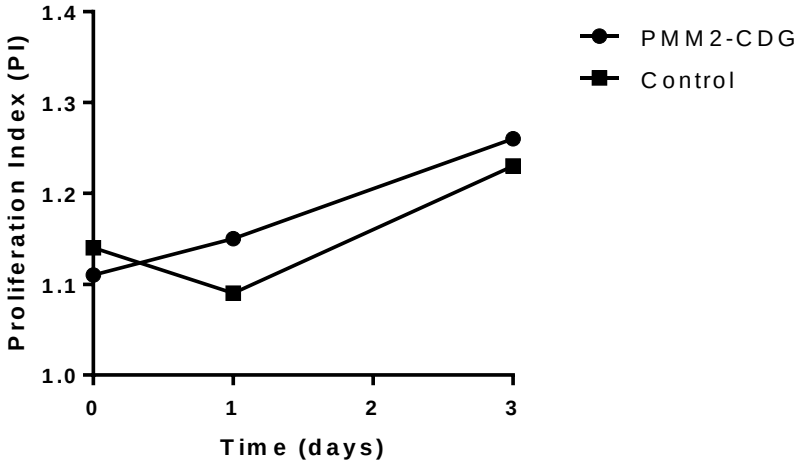

Figure 3.17 – PMM2-CDG cells proliferation capacity analysis by flow cytometry. CellTrace™ Far Red-stained PMM2-CDG and control PBMCs were stimulated with 10 nM PMA and 0,125 Ion μM during 3 days and the proliferation index (PI) were obtained over time.

The results in Figure 3.17 show that PMM2-CDG cells had more proliferating T cells than control cells, which support our previous observations that PMM2-CDG T cells seem to have more proliferating capacity to a mitogenic stimulation than his healthy sibling. To find more evidence to support this, we measured the genetic expression and secretion of activated T cell derived cytokines. In order to evaluate and quantify the cytokines genetic expression, RT-qPCR and the Livak and Schmittgen method were used.[140] As for the quantification of secreted cytokines, capture ELISA was performed. This technique works on the basis that the cytokines present in the sample are immobilized onto an antibody-bound solid surface and linked to an enzyme-linked antibody. When the enzyme substrate is added, the subsequent reaction produces a spectrophotometric measurable

product. To evaluate the proliferation capacity of T cells based on cytokine genetic expression and secretion, we wanted to analyze IL-4, IL-6, IL-10 and IFN-γ that are all derived cytokines from activated T cells. However, due to external factors related to reagents, only the evaluation of IFN-γ expression and secretion were assessed.

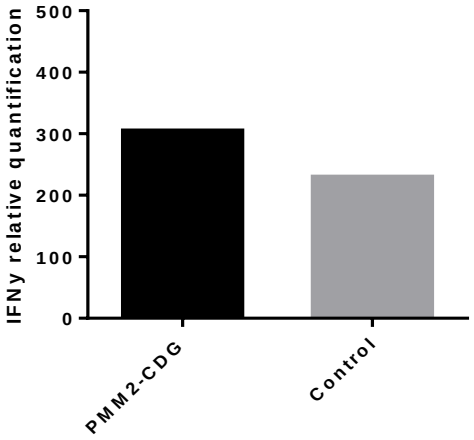

Figure 3.18 – IFN-γ genetic expression analysis by RT-qPCR. IFN-γ relative quantification of PMM2-CDG and control PBMCs, 24H after mitogenic stimulation.

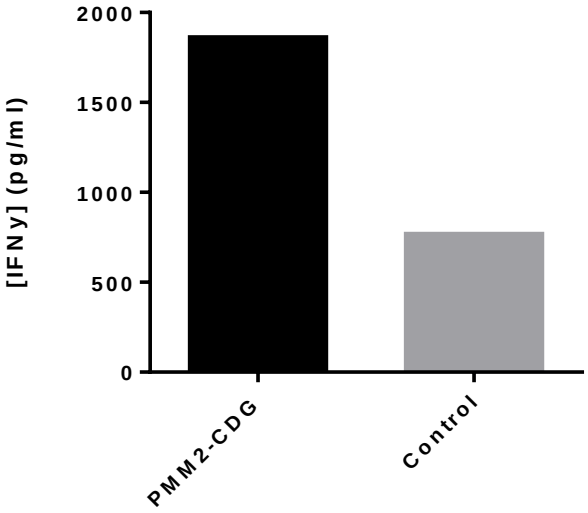

Figure 3.19 – IFN-γ production analysis by ELISA. Quantification of IFN-γ concentration on the extracellular medium of PMM2-CDG and control cells, 72H after mitogenic stimulation.

The analysis of the IFN-γ genetic expression showed that PMM2-CDG cells have a higher expression 24 H after mitogenic stimulation than control cells (Figure 3.18). In the same manner, the secretion of IFN-γ was also found to be increased in PMM2-CDG patient cells 72 H after the

stimulation (Figure 3.19). Both these results, support our previous observation that the PMM2-CDG patient T cells have a higher proliferation capacity than his healthy sibling. IFN-γ is indeed a cytokine derived from T_H1 cells upon activation to mediate cell-mediated immunity and responsible for the recruitment of effector cells.[8]

Defective N-glycosylation of the TCR has been shown to modulate T cell function, specifically by the increase in functional avidity in response to an antigen.[179] However, T cells stimulation with PMA and Ion is TCR-independent since it results in the direct activation of PKC.[22,23] Therefore, the higher proliferation capacity of PMM2-CDG T cells could be due to a basal pro-inflammatory state of these cells in which the threshold for T cell activation is lower. These pro-inflammatory state of type I CDGs have previously been proposed by Heyne *et al.* 1988 due to the accumulation of underglycosylated proteins in the ER.[129] Moreover, although we did not assess PKC expression levels, PKC could be overexpressed in PMM2-CDG cells compared to control cells providing a higher T cell activation signal upon phosphorylation by mitogenic stimulation. Thus, future experiments should include this particular assay to check for this possibility.

3.4. Part 3 – Exploratoty review of PROMs and ObsROMs for PMM2-CDG QoL evaluation

The third and final aim of this thesis was to do a literature review to find PROMs and ObsROMs to assess PMM2-CDG patients QoL and other instruments to evaluate parents and caregivers QoL. To select the articles adequate for our search, we conducted (1) a search in pubmed with pre-defined search terms (Appendix 6.3.1), and (2) after duplicates removal, we did a (3) SQL language selection, (4) a title and abstract-based selection and (5) a full-text based selection, considering our exclusion criteria. The initial search of Pubmed database yielded 48399 articles. Every result was uploaded onto Mendeley database from which 10461 duplicates were excluded. Mendeley is a relational database management system (RDBMS) being all the data structured and related.[180] The SQL language is a text-based language universilly accepted for accessing and manipulating data stored in a RDBMS, resorting to queries, i.e. a request of desired information.[181] Several queries were executed with different combination between terms, and the number of hits to each query is represented in Table 3.2.

Table 3.2 – Queries executed and number of results obtained.

Query	Nº of articles
1	40684
2	23809
3	23358
4	96

We can see in Table 3.2 that the number of articles diminished in each query attempt. Query 1 was found to be an unsuitable approach as it contained a lot of article repetitions. The results from query 2 and 3 were derived from combinations with only two keywords within the abstract and still retrieved an elevated number of articles. The 96 results from query 4 were derived from combinations with three keywords within in the abstract, one QoL related, other PMM2-CDG disease classification related and a PMM2-CDG related term. Therefore, the decrease in the article number is correlated with a higher specificity in the results. Considering this, query 4 was considered the more adequate one, and its 96 articles were selected to proceed with the title and abstract selection. To analyze the informatics selection using query 4, the correlation between the search terms were assessed. Therefore, we proceeded with the analysis of which were the QoL and PMM2-CDG symptoms terms that originated the 96 articles and which were the ones that did not retrieve any results. Figure 3.20 shows the correlation between QoL related terms and PMM2-CDG related terms. From the 18 QoL related terms used, only 9 retrieved one or more articles, being 'quality of life, 'measurement' and 'scale' the ones that derived more articles. Also, from the 39 symptoms included in our search only the 18 PMM2-CDG symptoms represented in Figure 3.20 were covered by our results.

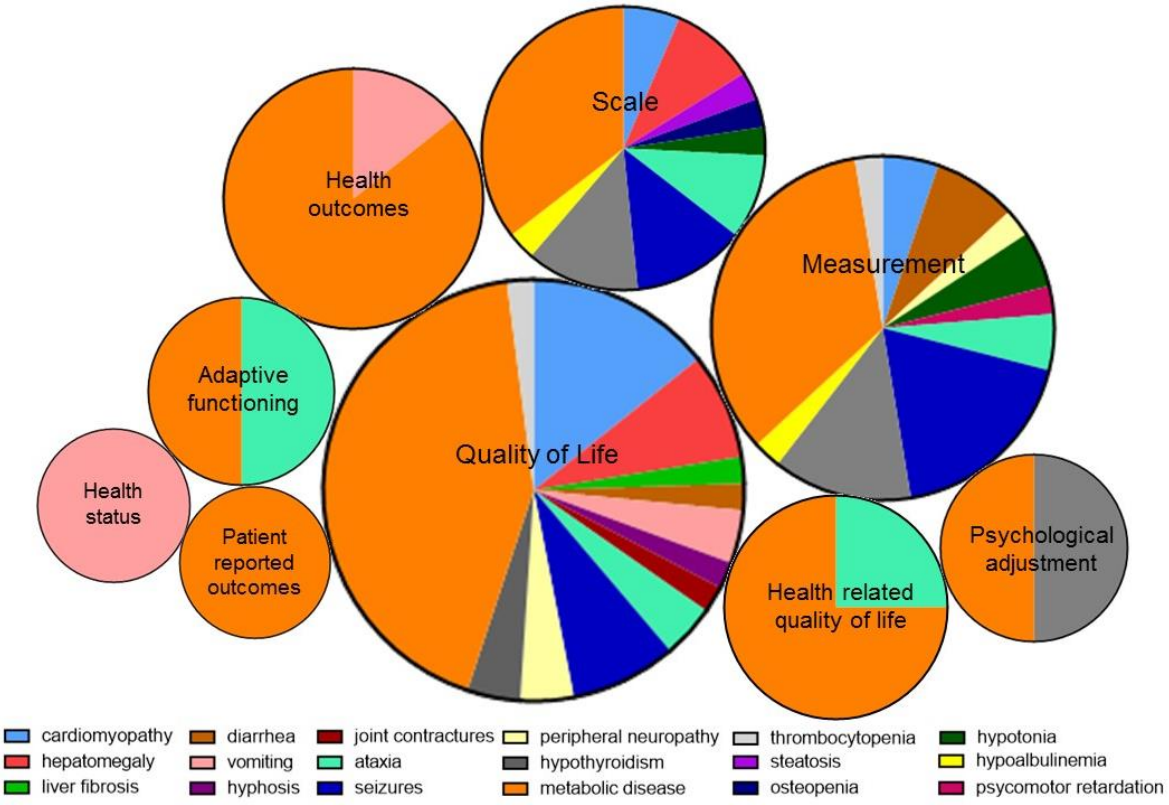

Figure 3.20 – Correlation between the QoL and PMM2-CDG symptoms keywords used in the search for the PROMs and ObsROMs literature review after SQL language selection. The area of the circles is proporcional to the number of articles retrived.

Another 115 articles were proposed by author referral, reference screening and by the parental QoL specific search. After the full-text based selection, 99 articles were included to do the review (Figure 3.21).

After data extraction, it was found that studies included self-, parent-, caregiver- and teacher-reports. Our search also revealed author built questionnaires. The instruments found assessed HrQoL (18 tools), psychological /adaptive adjustment and mental health (20 tools), specific symptoms (10 tools), gender identity (4 tools), treatment satisfaction (2 tools), self-concept and body image (2 tools), disease severity (1 tool) as well as coping (1 tool) (Figure 3.22). The great majority of instruments are generic instruments. In fact, a minority of the instruments found are disease-specific limited to the rare metabolic diseases Hirschsprung's disease/anorectal malformation, phenylketonuria, Fabry disease, Rett syndrome, mucopolysaccharidosis and primary ciliary dyskenesia. Generic instruments not only have the advantage of being cost-effective and applicable to every patient despite of the type of disease or the variety of clinical manifestations but also provide results comparable between patient groups with different diseases or healthy populations. However, they are not directed for the particular features within each condition. On the contrary, disease-specific instruments are responsive and sensitive but only confer the capability of making comparisons within the same patient group.[182] As disease-specific and generic instruments assess different aspects of HRQoL, it has been suggested to complement the administration of the first one with the latter.[183]

As for symptom-specific questionnaires, 10 instruments have bee identified (Figure 3.22). The clinical manifestations assessed by the included articles were limited to gastrointestinal affectation, nausea, epilepsy, pain, fatigue, sleep disturbance and manifestations of systemic sclerosis.

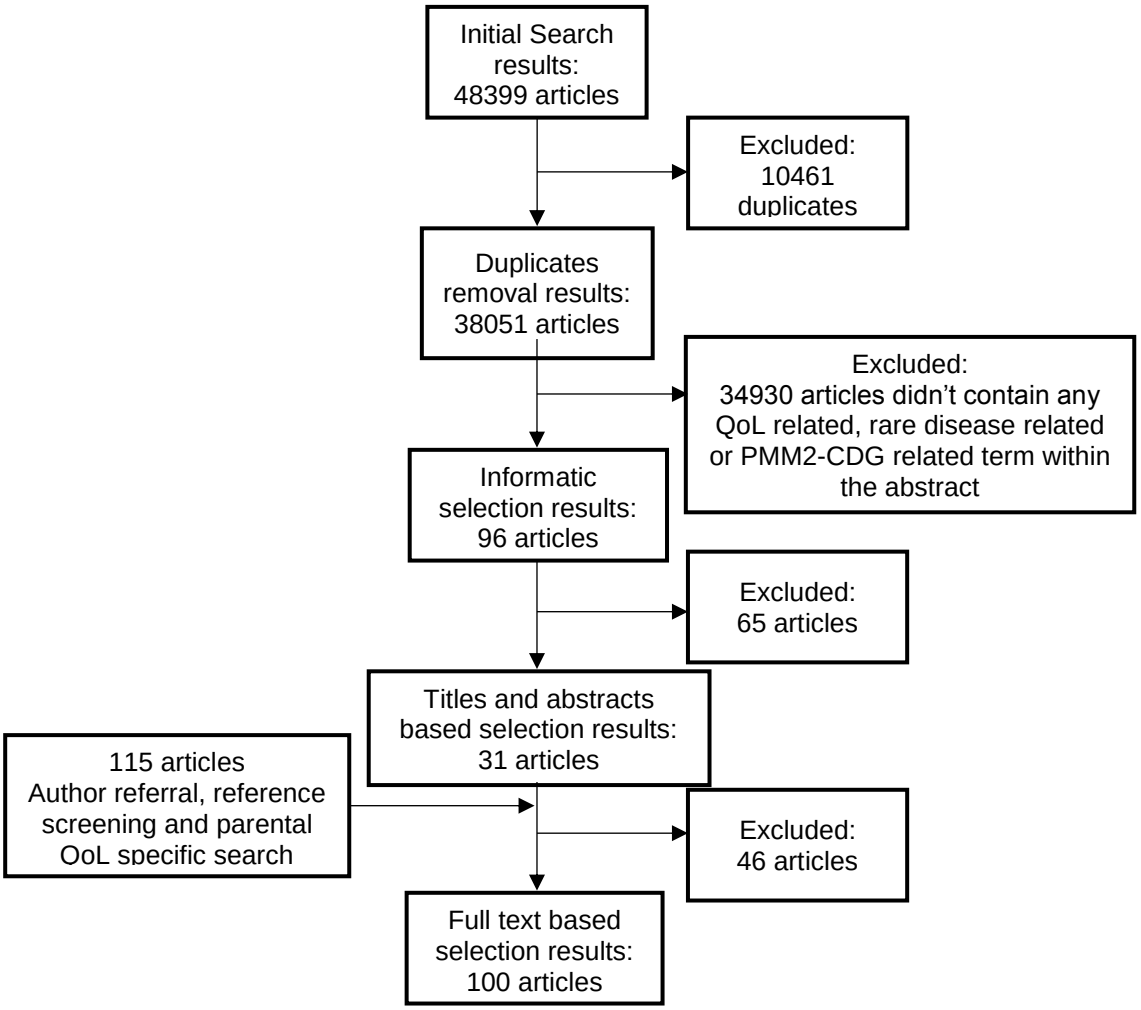

Figure 3.21 – Flowchart of the articles selection for the literature review of PROMs and ObsROMs, from initial search to final included articles.

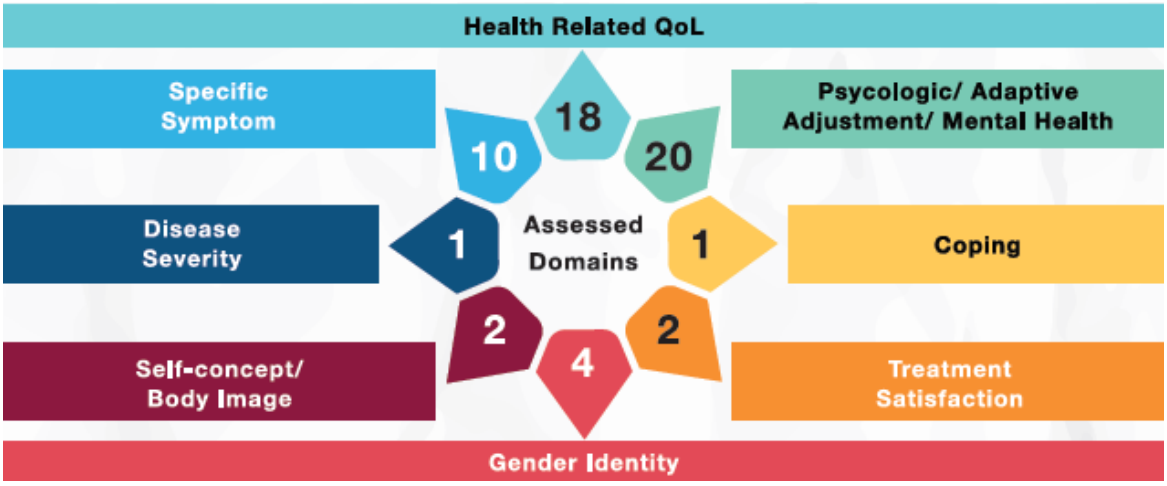

Figure 3.22 – Number of patients' QoL assessment PROMs and ObsROMs found depending on assessed domains.

Several studies were excluded because they did not report the use of any PROMs or ObsROMs but instead, they reported the use of clinician reported outcomes. One of these excluded studies reported the use of a tool specific for the clinician assessment of symptom severity and disease progression of CDG, the Nijmegen paediatric CDG rating scale.[184] Although specific for CDG, the outcomes measurement using this tool is not patient reported. Given the specificity of this instrument for CDG, we believe that this tool could be easily validated to a patient or observer reported context. In the same manner, PROMs and ObsROMs used for other rare metabolic diseases could be adapted and validated for the PMM2-CDG population as many of them have common clinical manisfestation due to the wide symptom diversity within PMM2-CDG. In fact, multiple entities, like United States Food and Drug Administration (FDA), have produced guidelines with standards to the development, assessment, implementation and analysis of PROMs.[185] The adaptation, development and validation of PROMs or ObsROMs is extremely needed, not only to integrate endpoints in future clinical trials for PMM2-CDG treatment, but also, for the establishment of these disease's natural history. Indeed, the unsderstanding of the PMM2-CDG immunological defects could greatly benefit benefit from the existence of a validated tool specifically directed at recording and analysing immunological parameters, alterations or symptoms.

Regarding parental QoL, we found 16 instruments among the included studies. These tools would be of great value in the evaluation of how is the QoL of PMM2-CDG patients' family affected while dealing with this disease in a daily basis and how would it change with therapy or future treatment.

4. Conclusions and Future Perspectives

During this project we reported that a ST6Gal-I transduced CRC cell line, SW48 ST6, express this enzyme and is able do secret this enzyme to the extracellular medium. Future studies should focus on assessing if the secreted enzyme is biologically active and if it has the capacity to extrinsically modulate DCs' functions as an event of cancer evasion of the immune response. Moreover, understanding the underlying mechanisms would be valuable in unravel the influence of α2,6 sialylation in the control of DCs' immune functions with possible application in the improvement of DC-based immunotherapies.

In the second part of this work, our attemp to immortalize a PMM2-CDG cell line using EBV-gene-containing lentiviral plasmids failed. However, we suggest that further attempts should include co-transduction with EBV and TERT genes, as well as other immortalization agents. Moreover, our results suggest that the PMM2-CDG patient enrolled in this study has a higher T cell proliferative capacity than his healthy sibling when challenged with a mitogen. We propose that it could be due to a basal pro-inflammatory state previously suggested by Heyne *et al* 1998 or even overexpression of PKC in PMM2-CDG cells compared to control cells, however we did not assess this events and future experiments should address this possibility.[129]Furthermore, more efforts should be made to assess the proliferation capacity in a cohort of PMM2-CDG patients to give significance to our results. Moreover, these studies should be performed in several time points of the patient lifespan as the immunological affectation of PMM2-CDG patients varies from child to adulthood, especially regarding susceptibility to infection. In the future, it should be created a biobank with PMM2-CDG patients' samples to try to unravel patterns regarding their immunological response. Besides PMM2-CDG patients' blood populations, T cell proliferation capacity and cytokine prodution, other immunological parameters should be adressed, for instance, (1) the assessment of immune cells' maturation markers, (2) the measurement of cytotoxic T cells activity based on the killing of virally infected cells, (3) the analysis of monocytes and neutrophils' phagocytic capacity and chemotactic response, (4) the measure of NK cells cytotoxic activity against tumor cells and (5) the measurement of circulating Igs' concentration. The establishment of the CDG natural history is extremelly needed, in particular with respect to the immunological involvemet, in order to develop new research strategies and find targeted and efficient treatment.

The increased efforts to try to find therapies and a cure for CDG are now highlighting a urgent unmet need: specific patient reported outcomes assessment tools to evaluate the QoL as a primary endpoint in clinical trials. We have been able to identify several patient reported tools used in the rare disease field. Taking in consideration that PMM2-CDG has a wide range of clinical manifestations, it has common symptoms to the identified diseases in the included studies. Thus, the same general PROMs or ObsROMs used for similar diseases may be also used to assess the QoL in PMM2-CDG patients. Future work should be focused on identifing the similarities between PMM2-CDG and the

identified diseases to propose the use or the adaptation of PROMs and ObsROMs. The existence of the international network CDG & Allies-PPAIN is an important advantage in this field, particularly, in the dissemination and patient recruitment needed for PROMs and ObsROMs validation in the CDG context. Regarding our innovative methodology to find the PROMs and ObsROMs, the experience derived from this work makes us suggest that the SQL language selection would be more reliable if the queries have been made against the articles' full-text insteat of the abstract. However, this strategy has revealed to be extremelly valuable in order to diminish the time and resources needed to do a literature review.

5. References

1. Abbas, A. K., Lichtman, A. H. & Pillai, S. *Cellular and molecular immunology*. (Elsevier, 2014).

2. Suresh, R. & Mosser, D. M. Pattern recognition receptors in innate immunity, host defense, and immunopathology. *Adv. Physiol. Educ.* **37,** 284–91 (2013).

3. Newton, K. & Dixit, V. M. Signaling in innate immunity and inflammation. *Cold Spring Harb. Perspect. Biol.* **4,** (2012).

4. Cray, C., Zaias, J. & Altman, N. H. Acute phase response in animals: a review. *Comp. Med.* **59,** 517–26 (2009).

5. Ahmed, R. & Gray, D. Immunological Memory and Protective Immunity: Understanding Their Relation. *Science (80-.).* **272,** 54–60 (1996).

6. Blum, J. S., Wearsch, P. A. & Cresswell, P. Pathways of Antigen Processing. *Annu. Rev. Immunol.* **31,** 443–473 (2013).

7. Andersen, M., Schrama, D., Thor Straten, P. & Becker, J. Cytotoxic T Cells. *J. Invest. Dermatol.* **126,** 32–41 (2006).

8. London, C. A., Abbas, A. K. & Kelso, A. Helper T cell subsets: Heterogeneity, functions and development. *Vet. Immunol. Immunopathol.* **63,** 37–44 (1998).

9. Mills, K. H. G. Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* **4,** 841–855 (2004).

10. Mescher, J. M. *et al.* Costimulation, and Signal 3 Cytokine Prolonged Exposure to Antigen, Development of Effector Function Require CD8 T Cell Clonal Expansion and CD8 T Cell Clonal Expansion and Development of Effector Function Require Prolonged Exposure to Antigen, Costimulation, and Signal 3 Cytokine. *J Immunol Ref.* **171,** 5165–5171 (2003).

11. Schroder, K. Interferon- : an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75,** 163–189 (2003).

12. Couper, K. N., Blount, D. G. & Riley, E. M. IL-10: The Master Regulator of Immunity to Infection. *J. Immunol.* **180,** 5771–5777 (2008).

13. Mannino, M. H. *et al.* The paradoxical role of IL-10 in immunity and cancer. *Cancer Lett.* **367,** 103–107 (2015).

14. Bradley, J. TNF-mediated inflammatory disease. *J. Pathol.* **214,** 149–160 (2008).

15. Sun, Y. *et al.* Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J. Recept. Signal Transduct.* **35,** 600–604 (2015).

16. Lis, H. & Sharon, N. Lectins: Carbohydrate-Specific Proteins That Mediate Cellular Recognition †. *Chem. Rev.* **98,** 637–674 (1998).

17. Hutchinson, P., Chadban, S. J., Atkins, R. C. & Holdsworth, S. R. Laboratory assessment of immune function in renal transplant patients. *Nephrol. Dial. Transplant.* **18,** 983–9 (2003).

18. Sakane, T., Steinberg, A. D. & Green, I. Studies of immune functions of patients with systemic lupus erythematosus. i. dysfunction of suppressor t-cell activity related to impaired generation of, rather than response to, suppressor cells. *Arthritis Rheum.* **21,** 657–664 (1978).

19. Cantrell, D. A. T-cell antigen receptor signal transduction. *Immunology* **105,** 369–74 (2002).

20. Touraine, J. L. Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. *J. Exp. Med.* **145,** 460–465 (1977).

21. Chopra, R. K. *et al.* Regulation of interleukin 2 and interleukin 2 receptor gene expression in human T cells: I. Effect of Ca2+-ionophore on phorbol myristate acetate co-stimulated cells. *Clin. Exp. Immunol.* **69,** 433–40 (1987).

22. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. & Nishizuka, Y. Protein kinase C as a possible receptor

protein of tumor-promoting phorbol esters. *J. Biol. Chem.* **258,** 11442–5 (1983).

23. Nishizuka, Y. Studies and perspectives of protein kinase C. *Science (80-.).* **233,** 305–312 (1986).

24. Chatila, T., Silverman, L., Miller, R. & Geha, R. Mechanisms of T cell activation by the calcium ionophore ionomycin. *J. Immunol.* **143,** 1283–9 (1989).

25. Scott, E. S., Malcomber, S. & O'Hare, P. Nuclear translocation and activation of the transcription factor NFAT is blocked by herpes simplex virus infection. *J. Virol.* **75,** 9955–65 (2001).

26. Hashimoto, S. *et al.* Mechanism of calcium ionophore and phorbol ester-induced T-cell activation. Accessory cell requirement for T-cell activation. *Scand. J. Immunol.* **33,** 393–403 (1991).

27. Varki, A. & Sharon, N. *Historical Background and Overview*. *Essentials of Glycobiology* (Cold Spring Harbor Laboratory Press, 2009).

28. Pinho, S. S. & Reis, C. A. Glycosylation in cancer: mechanisms and clinical implications. *Nat. Rev. Cancer* **15,** 540–555 (2015).

29. Öberg, F. *et al.* Glycosylation increases the thermostability of human aquaporin 10 protein. *J. Biol. Chem.* **286,** 31915–23 (2011).

30. Boscher, C., Dennis, J. W. & Nabi, I. R. Glycosylation, galectins and cellular signaling. *Curr. Opin. Cell Biol.* **23,** 383–92 (2011).

31. Somers, W. S., Tang, J., Shaw, G. D. & Camphausen, R. T. Insights into the Molecular Basis of Leukocyte Tethering and Rolling Revealed by Structures of P- and E-Selectin Bound to SLeX and PSGL-1. *Cell* **103,** 467–479 (2000).

32. Aebi, M. N-linked protein glycosylation in the ER. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833,** 2430–2437 (2013).

33. Schwarz, F. & Aebi, M. Mechanisms and principles of N-linked protein glycosylation. *Curr. Opin. Struct. Biol.* **21,** 576–582 (2011).

34. Bieberich, E. Synthesis, Processing, and Function of N-glycans in N-glycoproteins. *Adv. Neurobiol.* **9,** 47–70 (2014).

35. Freeze, H. H. & Aebi, M. Altered glycan structures: the molecular basis of congenital disorders of glycosylation. *Curr. Opin. Struct. Biol.* **15,** 490–498 (2005).

36. Steen, P. Van den, Rudd, P. M., Dwek, R. A. & Opdenakker, G. Concepts and Principles of O-Linked Glycosylation. *Crit. Rev. Biochem. Mol. Biol.* **33,** 151–208 (1998).

37. Ohtsubo, K. *et al.* Glycosylation in cellular mechanisms of health and disease. *Cell* **126,** 855–67 (2006).

38. Schedin-Weiss, S., Winblad, B. & Tjernberg, L. O. The role of protein glycosylation in Alzheimer disease. *FEBS J.* **281,** 46–62 (2014).

39. Lehri-Boufala, S. *et al.* New Roles of Glycosaminoglycans in α-Synuclein Aggregation in a Cellular Model of Parkinson Disease. *PLoS One* **10,** e0116641 (2015).

40. Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* **409,** 733–739 (2001).

41. Daniels, M. A., Hogquist, K. A. & Jameson, S. C. Sweet 'n' sour: the impact of differential glycosylation on T cell responses. *Nat. Immunol.* **3,** 903–910 (2002).

42. Rabinovich, G. A., van Kooyk, Y. & Cobb, B. A. Glycobiology of immune responses. *Ann. N. Y. Acad. Sci.* **1253,** 1–15 (2012).

43. van Kooyk, Y. & Rabinovich, G. A. Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat. Immunol.* **9,** 593–601 (2008).

44. Kaneko, Y. Anti-Inflammatory Activity of Immunoglobulin G Resulting from Fc Sialylation. *Science (80-.).* **313,** 670–673 (2006).

45. Chamorey, A.-L., Magné, N., Pivot, X. & Milano, G. Impact of glycosylation on the effect of cytokines. A special focus on oncology. *Eur. Cytokine Netw.* **13,** 154–60

46. Clark, M. C. & Baum, L. G. T cells modulate glycans on CD43 and CD45 during development and activation, signal regulation, and survival. *Ann. N. Y. Acad. Sci.* **1253,** 58–67 (2012).

47. Mitoma, J. *et al.* Critical functions of N-glycans in L-selectin-mediated lymphocyte homing and recruitment. *Nat. Immunol.* **8,** 409–418 (2007).

48. Varki, A. & Schauer, R. *Sialic Acids*. *Essentials of Glycobiology* (Cold Spring Harbor Laboratory Press, 2009).

49. Varki, A. Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. *Nature* **446,** 1023–1029 (2007).

50. Richards, A. A. *et al.* Sialic Acid Modification of Adiponectin Is Not Required for Multimerization or Secretion but Determines Half-Life in Circulation. *Mol. Endocrinol.* **24,** 229–239 (2010).

51. Traving, C. & Schauer, R. Structure, function and metabolism of sialic acids. *Cell. Mol. Life Sci.* **54,** 1330–1349 (1998).

52. Zhuo, Y. & Bellis, S. L. Emerging role of alpha2,6-sialic acid as a negative regulator of galectin binding and function. *J. Biol. Chem.* **286,** 5935–41 (2011).

53. Schauer, R. Sialic acids and their role as biological masks. *Trends Biochem. Sci.* **10,** 357–360 (1985).

54. Pekosz, A., Newby, C., Bose, P. S. & Lutz, A. Sialic acid recognition is a key determinant of influenza A virus tropism in murine trachea epithelial cell cultures. *Virology* **386,** 61–67 (2009).

55. Powell, L. D., Whiteheart, S. W. & Hart, G. W. Cell surface sialic acid influences tumor cell recognition in the mixed lymphocyte reaction. *J. Immunol.* **139,** 262–70 (1987).

56. Sperandio, M., Gleissner, C. A. & Ley, K. Glycosylation in immune cell trafficking. *Immunol. Rev.* **230,** 97–113 (2009).

57. Datta, A. K. & Paulson, J. C. The sialyltransferase "sialylmotif" participates in binding the donor substrate CMP-NeuAc. *J. Biol. Chem.* **270,** 1497–500 (1995).

58. Rao, F. V *et al.* Structural insight into mammalian sialyltransferases. *Nat. Struct. Mol. Biol.* **16,** 1186–1188 (2009).

59. Harduin-Lepers, A. *et al.* The human sialyltransferase family. *Biochimie* **83,** 727–737 (2001).

60. Li, Y. & Chen, X. Sialic acid metabolism and sialyltransferases: natural functions and applications. *Appl. Microbiol. Biotechnol.* **94,** 887–905 (2012).

61. Takashima, S., Tsuji, S. & Tsujimoto, M. Characterization of the second type of human beta-galactoside alpha 2,6-sialyltransferase (ST6Gal II), which sialylates Galbeta 1,4GlcNAc structures on oligosaccharides preferentially. Genomic analysis of human sialyltransferase genes. *J. Biol. Chem.* **277,** 45719–28 (2002).

62. Shibuya, N. *et al.* The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. *J. Biol. Chem.* **262,** 1596–601 (1987).

63. Kitagawa, H. & Paulson, J. C. Differential expression of five sialyltransferase genes in human tissues. *J. Biol. Chem.* **269,** 17872–8 (1994).

64. Chen, R. *et al.* Glycoproteomics Analysis of Human Liver Tissue by Combination of Multiple Enzyme Digestion and Hydrazide Chemistry Glycoproteomics Analysis of Human Liver Tissue by Combination of Multiple Enzyme Digestion and Hydrazide Chemistry. 651–661 (2009).

65. Kuhn, B. *et al.* The structure of human α-2,6-sialyltransferase reveals the binding mode of complex glycans. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **69,** 1826–1838 (2013).

66. Dall'Olio, F. *et al.* β-Galactoside β2,6 sialyltransferase in human colon cancer: Contribution of multiple transcripts to regulation of enzyme activity and reactivity with Sambucus nigra agglutinin. *Int. J. Cancer* **88,** 58–65 (2000).

67. Appenheimer, M. M. *et al.* Biologic contribution of P1 promoter-mediated expression of ST6Gal I sialyltransferase. *Glycobiology* **13,** 591–600 (2003).

68. Wen, D. X., Svensson, E. C. & Paulsons, J. C. Tissue-specific Alternative Splicing of the beta-Galactoside a2,6-Sialyltransferase Gene*. *J. Biol. Chem.* **267,** 2512–2518 (1992).

69. Hennet, T., Chui, D., Paulson, J. C. & Marth, J. D. Immune regulation by the ST6Gal sialyltransferase. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4504–9 (1998).

70. Marino, J. H. *et al.* Disruption of thymopoiesis in ST6Gal I-deficient mice. *Glycobiology* **18,** 719–26 (2008).

71. Nasirikenari, M., Segal, B. H., Ostberg, J. R., Urbasic, A. & Lau, J. T. Altered granulopoietic profile and exaggerated acute neutrophilic inflammation in mice with targeted deficiency in the sialyltransferase ST6Gal I. *Blood* **108,** (2006).

72. Nasirikenari, M. *et al.* Altered eosinophil profile in mice with ST6Gal-1 deficiency: an additional role for ST6Gal-1 generated by the P1 promoter in regulating allergic inflammation. *J. Leukoc. Biol.* **87,** 457–66 (2010).

73. Crespo, H. J. *et al.* Effect of sialic acid loss on dendritic cell maturation. *Immunology* **128,** e621-31 (2009).

74. Collins, B. E. *et al.* Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact. *Proc. Natl. Acad. Sci.* **101,** 6104–6109 (2004).

75. Kitazume, S. *et al.* Alzheimer's -secretase, -site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase. *Proc. Natl. Acad. Sci.* **98,** 13554–13559 (2001).

76. Legaigneur, P. *et al.* Exploring the acceptor substrate recognition of the human beta-galactoside alpha 2,6-sialyltransferase. *J. Biol. Chem.* **276,** 21608–17 (2001).

77. Lee-Sundlov, M. M. *et al.* Circulating blood and platelets supply glycosyltransferases that enable extrinsic extracellular glycosylation. *Glycobiology* **27,** 188–198 (2017).

78. Zhu, G. *et al.* Two soluble glycosyltransferases glycosylate less efficiently in vivo than their membrane bound counterparts. *Glycobiology* **8,** 831–40 (1998).

79. Jamieson, J. C., McCaffrey, G. & Harder, P. G. Sialyltransferase: a novel acute-phase reactant. *Comp. Biochem. Physiol. B.* **105,** 29–33 (1993).

80. Jones, M. B. *et al.* Role for Hepatic and Circulatory ST6Gal-1 Sialyltransferase in Regulating Myelopoiesis. *J. Biol. Chem.* **285,** 25009–25017 (2010).

81. Dougher, C. W. L. *et al.* The blood-borne sialyltransferase ST6Gal-1 is a negative systemic regulator of granulopoiesis. *J. Leukoc. Biol.* **102,** 507–516 (2017).

82. Nasirikenari, M., Veillon, L., Collins, C. C., Azadi, P. & Lau, J. T. Y. Remodeling of marrow hematopoietic stem and progenitor cells by non-self ST6Gal-1 sialyltransferase. *J. Biol. Chem.* **289,** 7178–89 (2014).

83. Jones, M. B. *et al.* B-cell–independent sialylation of IgG. *Proc. Natl. Acad. Sci.* **113,** 7207–7212 (2016).

84. Lee, M. M. *et al.* Platelets Support Extracellular Sialylation by Supplying the Sugar Donor Substrate. *J. Biol. Chem.* **289,** 8742–8748 (2014).

85. Wandall, H. H. *et al.* The origin and function of platelet glycosyltransferases. *Blood* **120,** 626–635 (2012).

86. Manhardt, C. T., Punch, P. R., Dougher, C. W. L. & Lau, J. T. Y. Extrinsic Sialylation is Dynamically Regulated by Systemic Triggers In Vivo. *J. Biol. Chem.* (2017). doi:10.1074/jbc.C117.795138

87. Stewart, B. W. & Wild, C. P. *World cancer report*. (2014).

88. Salem, M. S. Z. Cancer: Some genetic considerations. *Egypt. J. Med. Hum. Genet.* **16,** 1–10 (2015).

89. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144,** 646–674 (2011).

90. Liberti, M. V & Locasale, J. W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **41,** 211–8 (2016).

91. Swann, J. B. & Smyth, M. J. Immune surveillance of tumors. *J. Clin. Invest.* **117,** 1137–46 (2007).

92. Jacobs, J. F., Nierkens, S., Figdor, C. G., de Vries, I. J. M. & Adema, G. J. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol.* **13,** e32–e42 (2012).

93. Vinay, D. S. *et al.* Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Semin. Cancer Biol.* **35,** S185–S198 (2015).

94. Mantovani, A., Allavena, P., Sica, A. & Balkwill, F. Cancer-related inflammation. *Nature* **454,** 436–444 (2008).

95. Büll, C., den Brok, M. H. & Adema, G. J. Sweet escape: Sialic acids in tumor immune evasion. *Biochim. Biophys. Acta - Rev. Cancer* **1846,** 238–246 (2014).

96. Stowell, S. R., Ju, T. & Cummings, R. D. Protein glycosylation in cancer. *Annu. Rev. Pathol.* **10,** 473–510 (2015).

97. Varki, A., Kannagi, R. & Toole, B. P. *Glycosylation Changes in Cancer*. *Essentials of Glycobiology* (Cold Spring Harbor Laboratory Press, 2009).

98. Vajaria, B. N., Patel, K. R., Begum, R. & Patel, P. S. Sialylation: an Avenue to Target Cancer Cells. *Pathol. Oncol. Res.* **22,** 443–447 (2016).

99. Dall'Olio, F. & Chiricolo, M. Sialyltransferases in cancer. *Glycoconj. J.* **18,** 841–50

100. Bull, C., Stoel, M. A., den Brok, M. H. & Adema, G. J. Sialic Acids Sweeten a Tumor's Life. *Cancer Res.* **74,** 3199–3204 (2014).

101. Fearon, E. R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* **61,** 759–67 (1990).

102. De Rosa, M. *et al.* Genetics, diagnosis and management of colorectal cancer (Review). *Oncol. Rep.* **34,** 1087–1096 (2015).

103. O'Neil, B. H. & Goldberg, R. M. Innovations in Chemotherapy for Metastatic Colorectal Cancer: An Update of Recent Clinical Trials. *Oncologist* **13,** 1074–1083 (2008).

104. Holst, S., Wuhrer, M. & Rombouts, Y. *Glycosylation Characteristics of Colorectal Cancer*. *Advances in Cancer Research* **126,** (2015).

105. Swindall, A. F. *et al.* ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. *Cancer Res.* **73,** 2368–78 (2013).

106. Zhao, Y. *et al.* α2,6-Sialylation mediates hepatocellular carcinoma growth in vitro and in vivo by targeting the Wnt/β-catenin pathway. *Oncogenesis* **6,** e343 (2017).

107. Zhu, Y. *et al.* Suppression of a sialyltransferase by antisense DNA reduces invasiveness of human colon cancer cells in vitro. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1536,** 148–160 (2001).

108. Leibovitz, A. *et al.* Classification of human colorectal adenocarcinoma cell lines. *Cancer Res.* **36,** 4562–9 (1976).

109. Dall'olio, F., Malagolini, N., Stefano, G. Di, Ciambella, M. & Serafini-Cessi, F. α2, 6 sialylation ofN-acetyllactosaminic sequences in human colorectal cancer cell lines. Relationship with non-adherent growth. *Int. J. Cancer* **47,** 291–297 (1991).

110. Swindall, A. F. & Bellis, S. L. Sialylation of the Fas Death Receptor by ST6Gal-I Provides Protection against Fas-mediated Apoptosis in Colon Carcinoma Cells. *J. Biol. Chem.* **286,** 22982–22990 (2011).

111. Liu, Z. *et al.* ST6Gal-I Regulates Macrophage Apoptosis via α2-6 Sialylation of the TNFR1 Death Receptor. *J. Biol. Chem.* **286,** 39654–39662 (2011).

112. Park, J.-J. & Lee, M. Increasing the α 2,6 sialylation of glycoproteins may contribute to metastatic

spread and therapeutic resistance in colorectal cancer. *Gut Liver* **7,** 629–41 (2013).

113. Jaeken, J. & Matthijs, G. Congenital Disorders of Glycosylation: A Rapidly Expanding Disease Family. *Annu. Rev. Genomics Hum. Genet.* **8,** 261–278 (2007).

114. Freeze, H. H., Eklund, E. A., Ng, B. G. & Patterson, M. C. Neurological Aspects of Human Glycosylation Disorders. *Annu. Rev. Neurosci.* **38,** 105–125 (2015).

115. Marques-da-Silva, D. *et al.* Liver involvement in congenital disorders of glycosylation (CDG). A systematic review of the literature. *J. Inherit. Metab. Dis.* **40,** 195–207 (2017).

116. Coman, D., Irving, M., Kannu, P., Jaeken, J. & Savarirayan, R. The skeletal manifestations of the congenital disorders of glycosylation. *Clin. Genet.* **73,** 507–515 (2008).

117. Marques-da-Silva, D. *et al.* Cardiac complications of congenital disorders of glycosylation (CDG): a systematic review of the literature. *J. Inherit. Metab. Dis.* **40,** 657–672 (2017).

118. Monticelli, M., Ferro, T., Jaeken, J., dos Reis Ferreira, V. & Videira, P. A. Immunological aspects of congenital disorders of glycosylation (CDG): a review. *J. Inherit. Metab. Dis.* **39,** 765–780 (2016).

119. Blau, N., Duran, M., Gibson, K. M. & Dionisi-Vici, C. Physician's Guide to the Diagnosis, Treatment, and Follow-Up of Inherited Metabolic Diseases.

120. Jaeken, J., Hennet, T., Matthijs, G. & Freeze, H. H. CDG nomenclature: time for a change! *Biochim. Biophys. Acta* **1792,** 825–6 (2009).

121. Schaftingen, E. Van & Jaeken, J. Phosphomannomutase deficiency is a cause of carbohydrate-deficient glycoprotein syndrome type I. *FEBS Lett.* **377,** (1995).

122. OMIM - Online Mendelian Inheritance in Man. Available at: https://www.omim.org/entry/601785. (Accessed: 4th July 2017)

123. van de Loo, K. F. E. *et al.* Socio-emotional Problems in Children with CDG. *J. Inherit. Metab. Dis.* **11,** 139–148 (2013).

124. Monin, M.-L. *et al.* 29 French adult patients with PMM2-congenital disorder of glycosylation: outcome of the classical pediatric phenotype and depiction of a late-onset phenotype. *Orphanet J. Rare Dis.* **9,** 207 (2014).

125. Stromme, P., Maehlen, J., Strom, E. H. & Torvik, A. Postmortem Findings in Two Patients with the Carbohydrate-deficient Glycoprotein Syndrome. *Acta Paediatr.* **80,** 55–62 (1991).

126. Ong, B. B. *et al.* Retinal hemorrhages associated with meningitis in a child with a congenital disorder of glycosylation. *Forensic Sci. Med. Pathol.* **5,** 307–312 (2009).

127. Blank, C. *et al.* Recurrent infections and immunological dysfunction in congenital disorder of glycosylation Ia (CDG Ia). *J. Inherit. Metab. Dis.* **29,** 592–592 (2006).

128. Björklund, J. E. M., Stibler, H., Kristiansson, B., Johansson, S. G. O. & Magnusson, C. G. M. Immunoglobulin Levels in Patients with Carbohydrate-Deficient Glycoprotein Syndrome Type I. *Int. Arch. Allergy Immunol.* **114,** 116–119 (1997).

129. Heyne, K., Mayatepek, E., Walther, F., Weidinger, S. & Pahl, H. L. Pericardial effusion in glycanosis CDG type I (MIM 212 065): an inflammatory endoplasmic reticulum overload response? *Eur. J. Pediatr.* **157,** 168–9 (1998).

130. Noelle, V. *et al.* Unusual presentation of congenital disorder of glycosylation type 1a: congenital persistent thrombocytopenia, hypertrophic cardiomyopathy and hydrops-like aspect due to marked peripheral oedema. *Eur. J. Pediatr.* **164,** 223–226 (2005).

131. Sharma, N. S. Patient centric approach for clinical trials: Current trend and new opportunities. *Perspect. Clin. Res.* **6,** 134–8 (2015).

132. Fairclough, D. L. *Design and analysis of quality of life studies in clinical trials, Second Edition*. (Chapman and Hall/CRC, 2010).

133. Johnston, B. C. *et al.* Limited responsiveness related to the minimal important difference of patient-

reported outcomes in rare diseases. *J. Clin. Epidemiol.* **79,** 10–21 (2016).

134. Basch, E. & Bennett, A. V. Patient-Reported Outcomes in Clinical Trials of Rare Diseases. *J. Gen. Intern. Med.* **29,** 801–803 (2014).

135. Nelson, E. C. *et al.* Patient reported outcome measures in practice. *BMJ* **350,** (2015).

136. Verstovsek, S. *et al.* A Double-Blind, Placebo-Controlled Trial of Ruxolitinib for Myelofibrosis. *N. Engl. J. Med.* **366,** 799–807 (2012).

137. Crespo, H. J. *et al.* Effect of sialic acid loss on dendritic cell maturation. *Immunology* **128,** e621–e631 (2009).

138. Swindall, A. F. *et al.* ST6Gal-I Protein Expression Is Upregulated in Human Epithelial Tumors and Correlates with Stem Cell Markers in Normal Tissues and Colon Cancer Cell Lines. *Tumor Stem Cell Biol.* **73,** 2368–78 (2013).

139. Malagolini, N., Chiricolo, M., Marini, M. & Dall'Olio, F. Exposure of 2,6-sialylated lactosaminic chains marks apoptotic and necrotic death in different cell types. *Glycobiology* **19,** 172–181 (2008).

140. Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25,** 402–408 (2001).

141. Olio, F. D., Malagolini, N. & Serafini-Cessi, F. Enhanced CMP-NeuAc:Galβ1, 4GlcNAc-R α2, 6 sialyltransferase activity of human colon cancer xenografts in athymic nude mice and of xenograft-derived cell lines. *Int. J. Cancer* **50,** 325–330 (1992).

142. Mahmood, T. & Yang, P.-C. Western blot: technique, theory, and trouble shooting. *N. Am. J. Med. Sci.* **4,** 429–34 (2012).

143. Smith, B. J. SDS Polyacrylamide Gel Electrophoresis of Proteins. *Methods Mol Biol* **1,** 41–56 (1984).

144. Eisener-Dorman, A. F., Lawrence, D. A. & Bolivar, V. J. Cautionary insights on knockout mouse studies: the gene or not the gene? *Brain. Behav. Immun.* **23,** 318–24 (2009).

145. Schultz, M. J. *et al.* The Tumor-Associated Glycosyltransferase ST6Gal-I Regulates Stem Cell Transcription Factors and Confers a Cancer Stem Cell Phenotype. *Cancer Res.* **76,** 3978–3988 (2016).

146. MacPhee, D. J. Methodological considerations for improving Western blot analysis. *J. Pharmacol. Toxicol. Methods* **61,** 171–177 (2010).

147. Kroes, R. A. & Moskal, J. R. The role of DNA methylation in ST6Gal1 expression in gliomas. *Glycobiology* **26,** 1271–83 (2016).

148. Antony, P. *et al.* Epigenetic inactivation of ST6GAL1 in human bladder cancer. *BMC Cancer* **14,** 901 (2014).

149. Antequera, F., Boyes, J. & Bird, A. High levels of De Novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **62,** 503–514 (1990).

150. Verdone, L., Caserta, M. & Mauro, E. Di. Role of histone acetylation in the control of gene expression. *Biochem. Cell Biol.* **83,** 344–353 (2005).

151. Stacey, G., Doyle, A. & Ferro, M. A. Cell culture methods for in vitro toxicology. *Repr. from eell Biol. Toxicol.* **17,** (2001).

152. Verhoeyen, E., Costa, C. & Cosset, F.-L. *Lentiviral Vector Gene Transfer into Human T Cells*. *Methods in molecular biology (Clifton, N.J.)* **506,** (Humana Press, 2009).

153. Dolcetti, R. *et al.* Epstein-Barr virus and telomerase: from cell immortalization to therapy. *Infect. Agent. Cancer* **9,** 8 (2014).

154. Raab-Traub, N. *EBV-induced oncogenesis*. *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (Cambridge University Press, 2007).

155. Tosato, G. & Cohen, J. I. *Generation of Epstein-Barr Virus (EBV)-Immortalized B Cell Lines*. *Current*

Protocols in Immunology (John Wiley & Sons, Inc., 2007). doi:10.1002/0471142735.im0722s76

156. Nakayama, T. *et al.* Human B cells immortalized with Epstein-Barr virus upregulate CCR6 and CCR10 and downregulate CXCR4 and CXCR5. *J. Virol.* **76,** 3072–7 (2002).

157. Coelen, R. J., Jose, D. G. & May, J. T. The effect of hexadimethrine bromide (polybrene) on the infection of the primate retroviruses SSV 1/SSAV 1 and BaEV. *Arch. Virol.* **75,** 307–11 (1983).

158. abmTM. ViralPlus Transduction Enhancer. Available at: https://www.abmgood.com/ViralPlus-Lentivirus-Transduction-Efficiency-Enhancer.html.

159. Desfarges, S. & Ciuffi, A. in *Viruses: Essential Agents of Life* 147–175 (2012).

160. Nikitin, P. A. *et al.* An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. *Cell Host Microbe* **8,** 510–22 (2010).

161. Mallette, F. A., Gaumont-Leclerc, M.-F. & Ferbeyre, G. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev.* **21,** 43–8 (2007).

162. Sanchez, E. L. & Lagunoff, M. Viral activation of cellular metabolism. *Virology* **479–480,** 609–18 (2015).

163. Palsson-McDermott, E. M. & O'Neill, L. A. J. The Warburg effect then and now: From cancer to inflammatory diseases. *BioEssays* **35,** 965–973 (2013).

164. McFadden, K. *et al.* Metabolic stress is a barrier to Epstein–Barr virus-mediated B-cell immortalization. *Proc. Natl. Acad. Sci.* **113,** E782–E790 (2016).

165. Jeon, J.-P., Nam, H.-Y., Shim, S.-M. & Han, B.-G. Sustained viral activity of epstein-Barr virus contributes to cellular immortalization of lymphoblastoid cell lines. *Mol. Cells* **27,** 143–148 (2009).

166. BD Biosciences. Human and Mouse CD Marker Handbook. (2010).

167. Invitrogen. Cell concentrations in human and mouse samples. (2008).

168. Italiani, P. & Boraschi, D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. *Front. Immunol.* **5,** 514 (2014).

169. Mukherjee, R. *et al.* Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. *Sci. Rep.* **5,** (2015).

170. Zhou, W. *et al.* CellTrace™ Far Red; CellTracker™ Deep Red—long term live cell tracking for flow cytometry and fluorescence microscopy. *doi.org* 38 doi:10.14440/jbm.2016.113

171. Quah, B. J. C., Warren, H. S. & Parish, C. R. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat. Protoc.* **2,** 2049–2056 (2007).

172. Clarke, C. & Davies, S. *Immunomagnetic Cell Separation*. *Metastasis Research Protocols* **58,** (Humana Press, 2001).

173. Castagna, M. *et al.* Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257,** 7847–51 (1982).

174. Lymphocyte reactivity in vitro: I. Cellular reconstitution of purified lymphocyte response. *Cell. Immunol.* **1,** 207–218 (1970).

175. Lašťovička, J., Budinský, V., Špíšek, R. & Bartůňková, J. Assessment of lymphocyte proliferation: CFSE kills dividing cells and modulates expression of activation markers. *Cell. Immunol.* **256,** 79–85 (2009).

176. MacDonald, H. R. & Zaech, P. Light scatter analysis and sorting of cells activated in mixed leukocyte culture. *Cytometry* **3,** 55–58 (1982).

177. Videira, P. A. *et al.* ST3Gal.I sialyltransferase relevance in bladder cancer tissues and cell lines. *BMC Cancer* **9,** 357 (2009).

178. Zhang, X. & Mosser, D. M. Macrophage activation by endogenous danger signals. *J. Pathol.* **214,** 161–

78 (2008).

179. Kuball, J. *et al.* Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *J. Exp. Med.* **206,** 463–75 (2009).

180. Sumathi, S. & Esakkirajan, S. *Fundamentals of relational database management systems*. (Springer, 2007).

181. Jamison, D. C. *Structured Query Language (SQL) fundamentals. Current protocols in bioinformatics* (John Wiley and Sons, Inc., 2003). doi:10.1002/0471250953.bi0902s00

182. Wiebe, S., Guyatt, G., Weaver, B., Matijevic, S. & Sidwell, C. Comparative responsiveness of generic and specific quality-of-life instruments. *J. Clin. Epidemiol.* **56,** 52–60 (2003).

183. Assari, S., Lankarani, M. M., Montazeri, A., Soroush, M. R. & Mousavi, B. Are generic and disease-specific health related quality of life correlated? The case of chronic lung disease due to sulfur mustard. *J. Res. Med. Sci.* **14,** 285–90 (2009).

184. Achouitar, S. *et al.* Nijmegen paediatric CDG rating scale: a novel tool to assess disease progression. *J. Inherit. Metab. Dis.* **34,** 923–7 (2011).

185. FDA. *Guidance for Industry Patient-Reported Outcome Measures: Use in Medical Product Development to Support Labeling Claims Clinical/Medical Guidance for Industry Patient-Reported Outcome Measures: Use in Medical Product Development to Support Labeling Claims*. (2009).

186. Givan, A. L. *Flow Cytometry: First Principles*. (John Wiley & Sons, Inc., 2001). doi:10.1002/0471223948

187. Invitrogen. Attune ™ NxT Acoustic Focusing Cytometer. (2015).

188. Roederer, M. *Compensation in Flow Cytometry*. *Current Protocols in Cytometry* (John Wiley & Sons, Inc., 2002). doi:10.1002/0471142956.cy0114s22

189. Brown, T. A. *Gene Cloning and DNA Analysis: An Introduction*. (Wiley-Blackwell, 2012).

190. Zipper, H. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res.* **32,** e103–e103 (2004).

191. Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. Real time quantitative PCR. *Genome Res.* **6,** 986–94 (1996).

6. Appendix

6.1. Appendix 1: Solutions

Beads buffer: 0.5% BSA and 2 mM EDTA in PBS.

Blocking buffer/reagent dilutent (ELISA): 5% BSA in washing buffer for ELISA (see below).

Phosphate buffered saline (PBS) 1x: 1.47 mM of KH_2PO_4, 4.29 mM of $Na_2HPO_4.7H_2O$, 137 mM NaCl and 2.68 mM of KCl in distillated water (dH_2O), with a pH of 7.4.

Resolving Gel (12%): 3.3 ml of dH_2O, 4 ml of 30% acrylamide, 2.5 ml of 1.5 M Tris (pH 8.8), 100 µl of 10% SDS, 10 µl of TEMED and 100 µl of 10% APS, per gel.

Stacking Gel (4%): 1.8 ml of dH_2O, 402 µl of 30% acrylamide, 750 µl of 0.5 M Tris (pH 6.8), 30 µl of 10% SDS, 3 µl of TEMED and 30 µl of 10% APS, per gel.

TBS-T 0,1%: 15.4 mM of Tris-HCl and 137 mM of NaCl in dH_2O, with a pH of 7.6.

Washing buffer (ELISA): 0.05% (v/v) of Tween-20 in comercial PBS 1x.

6.2. Appendix 2: Techniques used in this project

6.2.1. Flow cytometry

Flow cytometry is a technique widely used in the Immunology field, allowing a multi-parameter analysis of single cells in a suspension. The principle behind this technique is that the flux of cells passing through a laser beam reflect, diffract or refract the light accordingly to their physical properties. The light that is detected in a small angle from the direction of the original beam is called forward scatter (FSC) signal and is proportional not only to the cross-sectional area of a particle but also with its refractive index. On the other side, the light that is scattered to the side (to an angle of 90º from the laser beam) is called side scatter (SSC) signal and is related to the cell's surface texture, internal structure and granularity. If the cells have intrinsic fluorescence or if they were previously stained with a fluorophore, the light beam can be converted into a different color and detected by specific photodetectors (or photomultiplier tubes – PTMs) with colored filters. The light that hits these PTMs is filtered, collected, converted into electrical signals, amplified and recorded as digital data. Each electrical signal originated by a cell that passes through the laser beam is known as an event[186]. One of the most important issues related with flow cytometry is related with the use of two or more fluorophores and the fact that they emit light over a range of wavelengths. Although the use of filters limit the range for each detector, there is often overlap between channels which impact the accuracy

and quality of the data acquired. In these cases, a correct compensation should be applied. Compensation stands for the process of mathematically correcting the fluorescence spillover between detectors, excluding the signal of any other fluorophores from a given channel except the correct one[187,188]. During this project, the Attune® Acoustic Focusing Cytometer (Applied Biosystems) was used. This apparatus has two lasers, red and blue with a wavelength of 638 nm and 488 nm, respectively, that enable the use of six different fluorophores at a time.

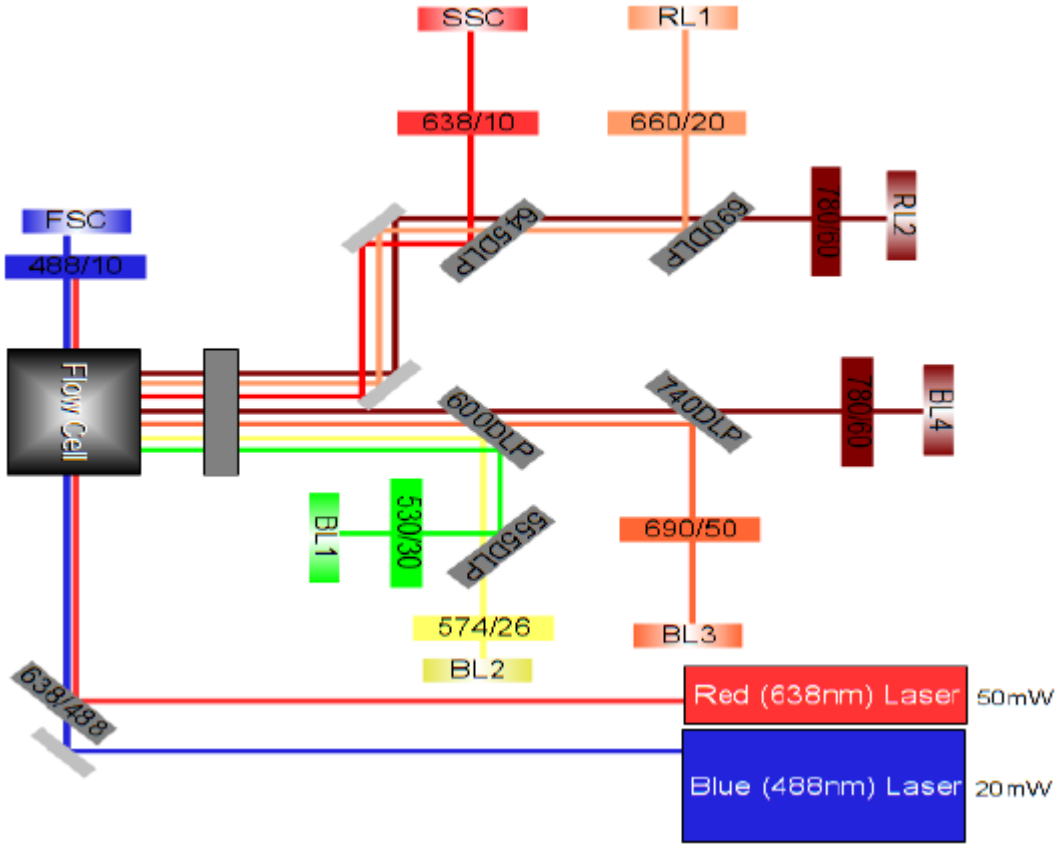

Figure 6.1 - Geometry of the Attune® Acoustic Focusing Cytometer (Applied Biosystems).

6.2.2. Real Time – quantitative Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a molecular biology technology that caused a profound impact on the scientific community. It has been used not only for DNA cloning and sequencing and gene expression analysis but also for diagnosis and monitoring of hereditary and infectious diseases. This technique resort to the ability of DNA polymerase to synthetize new strands of nucleic acid in the presence of a template, producing multiple copies in a rapid, inexpensive and simple way. The PCR reaction starts with the heating of double-stranded DNA that causes the separation of the two chains of DNA (denaturation), which will act as templates. Then, a diminution of the temperature

allows the primers to bond in complementary positions of the templates (annealing). These primers, also called probes, are small DNA sequences that delineate the starting and end position of the segment of DNA to amplify. The thermostable enzyme Taq DNA polymerase attach to the end of each primer and synthetize new chains of DNA complementary to the template sequence at an optimal working temperature of 74ºC (synthesis). The repetition of this cycle of denaturation-annealing-synthesis will produce millions of copies of the sequence of interest.[189] As the name points out, RT-PCR is an alternative to conventional PCR where the accumulation of amplification product is measured as the reaction occurs. In this thesis, SYBR Green and TaqMan chemistry were employed. The first relies on the use of a fluorescent dye that binds to dsDNA and increases its fluorescence. As the PCR progresses, SYBR Green dye will bind to the newly formed dsDNA molecules and consequently, the fluorescence intensity will be proportional to the amount of PCR product formed.[190] TaqMan chemistry evolves the use of probes linked to two fluorescent dyes. One fluorescent dye function as a reporter and its emission light is quenched by the second dye when the probe is intact. However, during the synthesis step of the PCR, the probe is cleaved by the nuclease activity of DNA polymerase and the quenching process is no longer viable, causing the emission light by the reporter dye to increase.[191] For gene expression quantification, the method introduced in 2001 by Livak and Schmittgen that describes the change in the expression of a certain gene in comparison to a exogenous control is universally used.[140]

6.3. Appendix 3: Search terms employed in the literature review

Search Strategy: 'Quality of life term' AND 'PMM2-CDG related term'

6.3.1. Patients Quality of Life

Quality of life terms (QoL)	PMM2-CDG related terms
Quality of Life measurement	**Disease classification**
Quality of Life	Rare disease/s
Disease-*specific* quality of life	Hereditary metabolic disease/s
Health-related quality of life	Orphan disease/s
Psychological adjustment	Metabolic rare disease/s
Adaptive functioning	Inborn errors of metabolism
Well-being	Inherited metabolic disease/s
Patient reported outcomes	Rare inherited disorder/s
Patient centered outcomes	Orphan inherited disease/s
Health outcomes	Rare inborn errors of metabolism
Health Status	**PMM2-CDG symptoms**
Scale	Pericardial effusion
Measurement	Cardiomyopathy
Proxy reported outcomes	Hepatomegaly
Disease-specific quality of life measures	Liver fibrosis
Generic -specific quality of life measures	Steatosis
Quality of life survey	Feeding problems
Quality of life assessment	Diarrhea
Quality of life questionnaire	Vomiting
Health care	Renal cysts
	Nephrotic syndrome
	Proximal tubulopathy
	Osteopenia
	Kyphosis

	Joint contractures
	Fat pads
	Hypotonia
	Psychomotor retardation
	Ataxia
	Hyporeflexia
	Stroke-like episodes
	Seizures
	Olivopontocerebellar hypoplasia
	Peripheral neuropathy
	Hypothyroidism
	Hypergonadotropic hypogonadism
	Antithrombin III deficiency
	Factor XI deficiency
	Thrombocytosis
	Decreased immunoglobulin A (IgA)
	Decreased immunoglobulin G (IgG)
	Proteinuria
	Hypocholesterolemia
	Hypoalbuminemia
	Elevated transaminases
	Metabolic disease
	Development delay
	Hypogammaglobulinemia
	Thrombocytopenia
	Hyperferritinemia

6.3.2. Parents Quality of Life

Quality of life terms (QoL)	PMM2-CDG related terms
1. Parents quality of life	Rare disease/s
	Hereditary metabolic disease/s
	Orphan disease
	Metabolic rare diseases
	Inborn errors of metabolism
	Inherited metabolic diseases
	Rare inherited disorders
	Orphan inherited diseases
	Rare inborn errors of metabolism

6.4. Appendix 4: Queries used in Navicat for SQL

Query 1

```
SELECT
Documents.pmid, Documents.title, Documents.publication, Documents.year, Documents.abstract
FROM
Documents
LEFT JOIN DocumentFolders ON Documents.id = DocumentFolders.documentId
LEFT JOIN Folders ON DocumentFolders.folderId = Folders.id
WHERE
 (Documents.abstract LIKE ('%quality of life measurement%')
 AND
(       (Documents.abstract LIKE '%rare disease%'
        OR Documents.abstract LIKE '%hereditary metabolic diseases%'
        OR Documents.abstract LIKE '%orphan disease%'
        OR Documents.abstract LIKE '%metabolic rare diseases%'
        OR Documents.abstract LIKE '%inborn errors of metabolism%'
        OR Documents.abstract LIKE '%inherited metabolic diseases%'
        OR Documents.abstract LIKE '%rare inherited disorders%'
        OR Documents.abstract LIKE '%orphan inherited diseases%'
        OR Documents.abstract LIKE '%rare inborn errors of metabolism%')
 OR
(       (Documents.abstract LIKE '%pericardial effusion%')
        OR (Documents.abstract LIKE '%cardiomyopathy%')
        OR (Documents.abstract LIKE '%hepatomegaly%')
        OR (Documents.abstract LIKE '%liver fibrosis%')
        OR (Documents.abstract LIKE '%steatosis%')
        OR (Documents.abstract LIKE '%feeding problems%')
        OR (Documents.abstract LIKE '%diarrhea%')
        OR (Documents.abstract LIKE '%vomiting%')
        OR (Documents.abstract LIKE '%renal cysts%')
        OR (Documents.abstract LIKE '%nephrotic syndrome%')
        OR (Documents.abstract LIKE '%proximal tubulopathy%')
        OR (Documents.abstract LIKE '%osteopenia%')
        OR (Documents.abstract LIKE '%kyphosis%')
        OR (Documents.abstract LIKE '%joint contractures%')
        OR (Documents.abstract LIKE '%fat pads%')
```

```
        OR (Documents.abstract LIKE '%hypotonia%')
        OR (Documents.abstract LIKE '%psychomotor retardation%')
        OR (Documents.abstract LIKE '%ataxia%')
        OR (Documents.abstract LIKE '%hyporeflexia%')
        OR (Documents.abstract LIKE '%stroke-like episodes%')
        OR (Documents.abstract LIKE '%seizures%')
        OR (Documents.abstract LIKE '%olivopontocerebellar hypoplasia%')
        OR (Documents.abstract LIKE '%peripheral neuropathy%')
        OR (Documents.abstract LIKE '%hypothyroidism%')
        OR (Documents.abstract LIKE '%hypergonadotropic hypogonadism%')
        OR (Documents.abstract LIKE '%antithrombin III deficiency%')
        OR (Documents.abstract LIKE '%factor XI deficiency%')
        OR (Documents.abstract LIKE '%thrombocytosis%')
        OR (Documents.abstract LIKE '%decreased immunoglobulin A%')
        OR (Documents.abstract LIKE '%decreased immunoglobulin B%')
        OR (Documents.abstract LIKE '%Proteinuria%')
        OR (Documents.abstract LIKE '%hypocholesterolemia%')
        OR (Documents.abstract LIKE '%hypoalbuminemia%')
        OR (Documents.abstract LIKE '%elevated transaminases%')
        OR (Documents.abstract LIKE '%metabolic diseases%')
        OR (Documents.abstract LIKE '%development delay%')
        OR (Documents.abstract LIKE '%hypogammaglobulinemia%')
        OR (Documents.abstract LIKE '%thrombocytopenia%')
        OR (Documents.abstract LIKE '%hyperferritinemia%')     ) ) )
ORDER BY
        DocumentFolders.folderId,
        Documents.id,
        DocumentFolders.documentId,
        Documents.pmid
```

Note: This querie was executed for each one of the QoL related terms.

Query 2

```
SELECT DISTINCT
Documents.pmid, Documents.title, Documents.publication, Documents.year, Documents.abstract
FROM
Documents
LEFT JOIN DocumentFolders ON Documents.id = DocumentFolders.documentId
LEFT JOIN Folders ON DocumentFolders.folderId = Folders.id
WHERE
((      Documents.abstract LIKE ('%Quality of life measurement%')
        OR Documents.abstract LIKE ('%Quality of life%')
        OR Documents.abstract LIKE ('%Disease-specific quality of life%')
        OR Documents.abstract LIKE ('%Health-related quality of life%')
        OR Documents.abstract LIKE ('%Psychological adjustment%')
        OR Documents.abstract LIKE ('%Adaptive functioning%')
        OR Documents.abstract LIKE ('%Well-being%')
        OR Documents.abstract LIKE ('%Patient reported outcomes%')
        OR Documents.abstract LIKE ('%Patient centered outcomes%')
        OR Documents.abstract LIKE ('%Health outcomes%')
        OR Documents.abstract LIKE ('%Health status%')
        OR Documents.abstract LIKE ('%Scale%')
        OR Documents.abstract LIKE ('%Measurement%')
        OR Documents.abstract LIKE ('%Proxy reported outcomes%')
        OR Documents.abstract LIKE ('%Disease-specific quality of life measures%')
        OR Documents.abstract LIKE ('%Generic-specific quality of life measures%')
        OR Documents.abstract LIKE ('%Quality of life survey%')
        OR Documents.abstract LIKE ('%Quality of life assessment%')    )
AND
(       (Documents.abstract LIKE '%rare disease%'
        OR Documents.abstract LIKE '%hereditary metabolic diseases%'
        OR Documents.abstract LIKE '%orphan disease%'
        OR Documents.abstract LIKE '%metabolic rare diseases%'
        OR Documents.abstract LIKE '%inborn errors of metabolism%'
        OR Documents.abstract LIKE '%inherited metabolic diseases%'
        OR Documents.abstract LIKE '%rare inherited disorders%'
        OR Documents.abstract LIKE '%orphan inherited diseases%'
        OR Documents.abstract LIKE '%rare inborn errors of metabolism%')
OR
(       (Documents.abstract LIKE '%pericardial effusion%')
```

```
OR (Documents.abstract LIKE '%cardiomyopathy%')
OR (Documents.abstract LIKE '%hepatomegaly%')
OR (Documents.abstract LIKE '%liver fibrosis%')
OR (Documents.abstract LIKE '%steatosis%')
OR (Documents.abstract LIKE '%feeding problems%')
OR (Documents.abstract LIKE '%diarrhea%')
OR (Documents.abstract LIKE '%vomiting%')
OR (Documents.abstract LIKE '%renal cysts%')
OR (Documents.abstract LIKE '%nephrotic syndrome%')
OR (Documents.abstract LIKE '%proximal tubulopathy%')
OR (Documents.abstract LIKE '%osteopenia%')
OR (Documents.abstract LIKE '%kyphosis%')
OR (Documents.abstract LIKE '%joint contractures%')
OR (Documents.abstract LIKE '%fat pads%')
OR (Documents.abstract LIKE '%hypotonia%')
OR (Documents.abstract LIKE '%psychomotor retardation%')
OR (Documents.abstract LIKE '%ataxia%')
OR (Documents.abstract LIKE '%hyporeflexia%')
OR (Documents.abstract LIKE '%stroke-like episodes%')
OR (Documents.abstract LIKE '%seizures%')
OR (Documents.abstract LIKE '%olivopontocerebellar hypoplasia%')
OR (Documents.abstract LIKE '%peripheral neuropathy%')
OR (Documents.abstract LIKE '%hypothyroidism%')
OR (Documents.abstract LIKE '%hypergonadotropic hypogonadism%')
OR (Documents.abstract LIKE '%antithrombin III deficiency%')
OR (Documents.abstract LIKE '%factor XI deficiency%')
OR (Documents.abstract LIKE '%thrombocytosis%')
OR (Documents.abstract LIKE '%decreased immunoglobulin A%')
OR (Documents.abstract LIKE '%decreased immunoglobulin B%')
OR (Documents.abstract LIKE '%Proteinuria%')
OR (Documents.abstract LIKE '%hypocholesterolemia%')
OR (Documents.abstract LIKE '%hypoalbuminemia%')
OR (Documents.abstract LIKE '%elevated transaminases%')
OR (Documents.abstract LIKE '%metabolic diseases%')
OR (Documents.abstract LIKE '%development delay%')
OR (Documents.abstract LIKE '%hypogammaglobulinemia%')
OR (Documents.abstract LIKE '%thrombocytopenia%')
OR (Documents.abstract LIKE '%hyperferritinemia%')     ) ))
```

```
ORDER BY
        DocumentFolders.folderId,
        Documents.id,
        DocumentFolders.documentId,
        Documents.pmid
```

Query 3

```
SELECT DISTINCT
Documents.pmid, Documents.title, Documents.publication, Documents.year, Documents.abstract
FROM
Documents
LEFT JOIN DocumentFolders ON Documents.id = DocumentFolders.documentId
LEFT JOIN Folders ON DocumentFolders.folderId = Folders.id
WHERE
(       (Documents.abstract LIKE '%Quality of life measurement%'
        OR Documents.abstract LIKE '%Quality of life%'
        OR Documents.abstract LIKE '%Disease-specific quality of life%'
        OR Documents.abstract LIKE '%Health-related quality of life%'
        OR Documents.abstract LIKE '%Psychological adjustment%'
        OR Documents.abstract LIKE '%Adaptive functioning%'
        OR Documents.abstract LIKE '%Well-being%'
        OR Documents.abstract LIKE '%Patient reported outcomes%'
        OR Documents.abstract LIKE '%Patient centered outcomes%'
        OR Documents.abstract LIKE '%Health outcomes%'
        OR Documents.abstract LIKE '%Health status%'
        OR Documents.abstract LIKE '%Scale%'
        OR Documents.abstract LIKE '%Measurement%'
        OR Documents.abstract LIKE '%Proxy reported outcomes%'
        OR Documents.abstract LIKE '%Disease-specific quality of life measures%'
        OR Documents.abstract LIKE '%Generic-specific quality of life measures%'
        OR Documents.abstract LIKE '%Quality of life survey%'
        OR Documents.abstract LIKE '%Quality of life assessment%'
        OR Documents.abstract LIKE '%rare disease%'
        OR Documents.abstract LIKE '%hereditary metabolic diseases%'
        OR Documents.abstract LIKE '%orphan disease%'
        OR Documents.abstract LIKE '%metabolic rare diseases%'
        OR Documents.abstract LIKE '%inborn errors of metabolism%'
        OR Documents.abstract LIKE '%inherited metabolic diseases%'
```

```
        OR Documents.abstract LIKE '%rare inherited disorders%'
        OR Documents.abstract LIKE '%orphan inherited diseases%'
        OR Documents.abstract LIKE '%rare inborn errors of metabolism%')
AND
        (Documents.abstract LIKE '%pericardial effusion%'
        OR Documents.abstract LIKE '%cardiomyopathy%'
        OR Documents.abstract LIKE '%hepatomegaly%'
        OR Documents.abstract LIKE '%liver fibrosis%'
        OR Documents.abstract LIKE '%steatosis%'
        OR Documents.abstract LIKE '%feeding problems%'
        OR Documents.abstract LIKE '%diarrhea%'
        OR Documents.abstract LIKE '%vomiting%'
        OR Documents.abstract LIKE '%renal cysts%‘
        OR Documents.abstract LIKE '%nephrotic syndrome%'
        OR Documents.abstract LIKE '%proximal tubulopathy%'
        OR Documents.abstract LIKE '%osteopenia%'
        OR Documents.abstract LIKE '%kyphosis%'
        OR Documents.abstract LIKE '%joint contractures%'
        OR Documents.abstract LIKE '%fat pads%'
        OR Documents.abstract LIKE '%hypotonia%'
        OR Documents.abstract LIKE '%psychomotor retardation%'
        OR Documents.abstract LIKE '%ataxia%'
        OR Documents.abstract LIKE '%hyporeflexia%'
        OR Documents.abstract LIKE '%stroke-like episodes%'
        OR Documents.abstract LIKE '%seizures%'
        OR Documents.abstract LIKE '%olivopontocerebellar hypoplasia%'
        OR Documents.abstract LIKE '%peripheral neuropathy%'
        OR Documents.abstract LIKE '%hypothyroidism%'
        OR Documents.abstract LIKE '%hypergonadotropic hypogonadism%'
        OR Documents.abstract LIKE '%antithrombin III deficiency%'
        OR Documents.abstract LIKE '%factor XI deficiency%'
        OR Documents.abstract LIKE '%thrombocytosis%'
        OR Documents.abstract LIKE '%decreased immunoglobulin A%'
        OR Documents.abstract LIKE '%decreased immunoglobulin B%'
        OR Documents.abstract LIKE '%Proteinuria%'
        OR Documents.abstract LIKE '%hypocholesterolemia%'
        OR Documents.abstract LIKE '%hypoalbuminemia%'
        OR Documents.abstract LIKE '%elevated transaminases%'
```

```
        OR Documents.abstract LIKE '%metabolic diseases%'
        OR Documents.abstract LIKE '%development delay%'
        OR Documents.abstract LIKE '%hypogammaglobulinemia%'
        OR Documents.abstract LIKE '%thrombocytopenia%'
        OR Documents.abstract LIKE '%hyperferritinemia%')   )
 ORDER BY
         DocumentFolders.folderId,
         Documents.id,
         DocumentFolders.documentId,
         Documents.pmid
```

Query 4

```
SELECT DISTINCT
Documents.pmid, Documents.title, Documents.publication, Documents.year, Documents.abstract
FROM
Documents
LEFT JOIN DocumentFolders ON Documents.id = DocumentFolders.documentId
LEFT JOIN Folders ON DocumentFolders.folderId = Folders.id
WHERE
        (Documents.abstract LIKE '%Quality of life measurement%'
        OR Documents.abstract LIKE '%Quality of life%'
        OR Documents.abstract LIKE '%Disease-specific quality of life%'
        OR Documents.abstract LIKE '%Health-related quality of life%'
        OR Documents.abstract LIKE '%Psychological adjustment%'
        OR Documents.abstract LIKE '%Adaptive functioning%'
        OR Documents.abstract LIKE '%Well-being%'
        OR Documents.abstract LIKE '%Patient reported outcomes%'
        OR Documents.abstract LIKE '%Patient centered outcomes%'
        OR Documents.abstract LIKE '%Health outcomes%'
        OR Documents.abstract LIKE '%Health status%'
        OR Documents.abstract LIKE '%Scale%'
        OR Documents.abstract LIKE '%Measurement%'
        OR Documents.abstract LIKE '%Proxy reported outcomes%'
        OR Documents.abstract LIKE '%Disease-specific quality of life measures%'
        OR Documents.abstract LIKE '%Generic-specific quality of life measures%'
        OR Documents.abstract LIKE '%Quality of life survey%'
        OR Documents.abstract LIKE '%Quality of life assessment%')
AND
```

```
        (Documents.abstract LIKE '%rare disease%'
        OR Documents.abstract LIKE '%hereditary metabolic disease%'
        OR Documents.abstract LIKE '%orphan disease%'
        OR Documents.abstract LIKE '%metabolic rare diseases%'
        OR Documents.abstract LIKE '%inborn errors of metabolism%'
        OR Documents.abstract LIKE '%inherited metabolic disease%'
        OR Documents.abstract LIKE '%rare inherited disorder%'
        OR Documents.abstract LIKE '%orphan inherited disease%'
        OR Documents.abstract LIKE '%rare inborn errors of metabolism%')
AND
        (Documents.abstract LIKE '%pericardial effusion%'
        OR Documents.abstract LIKE '%cardiomyopathy%'
        OR Documents.abstract LIKE '%hepatomegaly%'
        OR Documents.abstract LIKE '%liver fibrosis%'
        OR Documents.abstract LIKE '%steatosis%'
        OR Documents.abstract LIKE '%feeding problem%'
        OR Documents.abstract LIKE '%diarrhea%'
        OR Documents.abstract LIKE '%vomiting%'
        OR Documents.abstract LIKE '%renal cysts%'
        OR Documents.abstract LIKE '%nephrotic syndrome%'
        OR Documents.abstract LIKE '%proximal tubulopathy%'
        OR Documents.abstract LIKE '%osteopenia%'
        OR Documents.abstract LIKE '%kyphosis%'
        OR Documents.abstract LIKE '%joint contracture%'
        OR Documents.abstract LIKE '%fat pads%'
        OR Documents.abstract LIKE '%hypotonia%'
        OR Documents.abstract LIKE '%psychomotor retardation%'
        OR Documents.abstract LIKE '%ataxia%'
        OR Documents.abstract LIKE '%hyporeflexia%'
        OR Documents.abstract LIKE '%stroke-like episode%'
        OR Documents.abstract LIKE '%seizure%'
        OR Documents.abstract LIKE '%olivopontocerebellar hypoplasia%'
        OR Documents.abstract LIKE '%peripheral neuropathy%'
        OR Documents.abstract LIKE '%hypothyroidism%'
        OR Documents.abstract LIKE '%hypergonadotropic hypogonadism%'
        OR Documents.abstract LIKE '%antithrombin III deficiency%'
        OR Documents.abstract LIKE '%factor XI deficiency%'
        OR Documents.abstract LIKE '%thrombocytosis%'
```

```
        OR Documents.abstract LIKE '%decreased immunoglobulin A%'
        OR Documents.abstract LIKE '%decreased immunoglobulin B%'
        OR Documents.abstract LIKE '%Proteinuria%'
        OR Documents.abstract LIKE '%hypocholesterolemia%'
        OR Documents.abstract LIKE '%hypoalbuminemia%'
        OR Documents.abstract LIKE '%elevated transaminases%'
        OR Documents.abstract LIKE '%metabolic disease%'
        OR Documents.abstract LIKE '%development delay%'
        OR Documents.abstract LIKE '%hypogammaglobulinemia%'
        OR Documents.abstract LIKE '%thrombocytopenia%'
        OR Documents.abstract LIKE '%hyperferritinemia%')
ORDER BY
        DocumentFolders.folderId,
        Documents.id,
        DocumentFolders.documentId,
        Documents.pmid
```