DEPARTAMENT OF LIFE SCIENCES

TOWARDS THERAPEUTIC APPROACHES FOR HUMAN GLYCOSYLATION DISORDERS

FROM TRANSCRIPTOMIC AND IMMUNOLOGICAL CHARACTERIZATION TO PATIENT-CENTRED OUTCOMES RESEARCH

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MSc Biochemistry for Health

DOCTORATE IN BIOLOGY

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DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Towards therapeutic approaches for human glycosylation disorders: from transcriptomic and immunological characterization to patient-centred outcomes research

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."
– Marie Curie

ABSTRACT

Glycosylation is a post-translational modification that occurs ubiquitously in the human body and essential for all biological and physiological body functions. An impaired or altered glycosylation process affects homeostasis, leading to diseases like congenital disorders of glycosylation (CDG), cancer and immunological disbalance.

This thesis focuses on two glycosylation disorders, namely the ultra-rare phosphomannomutase 2 (PMM2)-CDG and the low incident triple negative breast cancer (TNBC). These complex, long-term conditions are associated with fundamental challenges, poor patient outcomes and reduced well-being. In this context, patient-centric research constitutes an opportunity to generate inclusive methodologies and meaningful results answering urgent community needs.

The aim of this thesis was to implement a public involvement framework and devise patient-oriented research on community-identified topics. Using a comprehensive approach of transcriptomics and validation techniques, this thesis validates patient-derived fibroblasts as a cellular model to study immunopathology, given the scarcity of other models, particularly in PMM2-CDG. This work allowed to proceed with further studies to unveil the mechanisms behind immunological involvement in PMM2-CDG, encompassing highly impactful and life-threatening manifestations. Glycosylation-related mechanistic defects were identified, specifically, dysregulated immune receptor downstream signalling and immune effectors, highlighting potential therapeutic targets and interconnections with other affected systems. Importantly, patient-reported outcomes measures that evaluate health related quality of life for specific PMM2-CDG symptoms were identified and analysed. This is a contribution to an urgent need that can aid in future clinical research stages, enabling the accurate measurement of targeted therapeutic benefits of investigational drugs. Lastly, using a methodology based in three different models, the role of a glycan epitope was characterized in TNBC which correlates with disease aggressiveness, contributing to patient stratification and the potential development of more targeted therapies.

In conclusion, this thesis illustrates the benefits of adopting patient-centric approaches in research, combining scientific progress in unexplored and urgent topics with desired and meaningful research outcomes for the community.

Keywords: People-centric research; Glycosylation; PMM2-CDG; Triple-negative breast cancer; Immunological involvement; Patient-reported outcomes

RESUMO

A glicosilação é uma modificação pós-translacional que ocorre ubiquamente no corpo humano e é essencial para todas as funções biológicas e fisiológicas do corpo. Um processo de glicosilação deficiente ou alterado afecta a homeostase, conduzindo a doenças como as doenças congénitas da glicosilação (CDG), o cancro e desequilíbrio imunológico.

Esta tese centra-se em duas doenças da glicosilação, nomeadamente a doença ultra-rara fosfomanomutase 2 (PMM2)-CDG e o cancro da mama triplo-negativo (TNBC), com baixa incidência. Estas doenças complexas e de longa duração estão associadas a desafios fundamentais, a maus resultados clinicos para os doentes e a um menor bem-estar. Neste contexto, a investigação centrada no doente constitui uma oportunidade para gerar metodologias inclusivas e resultados significativos que respondam a necessidades urgentes da comunidade.

O objetivo desta tese foi implementar uma *framework* de envolvimento público e conceber investigação orientada para os doentes sobre tópicos identificados pela comunidade. Utilizando uma abordagem abrangente de transcriptómica e técnicas de validação, esta tese valida os fibroblastos derivados de doentes como um modelo celular para estudar imunopatologias, dada a escassez de outros modelos, particularmente em PMM2-CDG. Este trabalho permitiu prosseguir com estudos adicionais para desvendar os mecanismos subjacentes ao envolvimento imunológico em PMM2-CDG, que abrange manifestações altamente impactantes e potencialmente fatais. Foram identificados defeitos mecanisticos relacionados com a glicosilação, especificamente, uma sinalização a jusante de um receptor imunologico desregulada bem como os seus efectores imunitários, destacando potenciais alvos terapêuticos e interconexões com outros sistemas afectados. É importante salientar que foram identificados e analisados *patient-reported outcome measures* que medem a qualidade de vida relacionada com a saúde para sintomas específicos de PMM2-CDG. Esta é uma contribuição para uma necessidade urgente que pode ajudar em futuras fases de investigação clínica, permitindo a medição exacta dos benefícios terapêuticos específicos dos medicamentos em investigação. Por último, utilizando uma metodologia baseada em três modelos diferentes, foi caracterizado o papel de um epítopo de glicano no TNBC que se

correlaciona com a agressividade da doença, contribuindo para a estratificação dos doentes e para o potencial desenvolvimento de terapias mais direccionadas.

Em conclusão, esta tese ilustra os benefícios da adoção de abordagens centradas no doente na investigação, combinando o progresso científico em tópicos inexplorados e urgentes com resultados de investigação desejados e significativos para a comunidade.

Palavras-chave: Investigação centrada nas pessoas; Glicosilação; PMM2-CDG; Cancro da mama triplo-negativo (TNBC); Envolvimento imunológico; Patient-reported outcomes

OUTPUTS

Scientific peer-reviewed publications as first author

Pascoal C, Brasil S, Francisco R, Marques-da-Silva D, Rafalko A, Jaeken J, Videira PA, Barros L, dos Reis Ferreira V, Patient and observer reported outcome measures to evaluate health-related quality of life in inherited metabolic diseases: a scoping review, Orphanet J Rare Dis. 2018 Nov,13:215. doi: 10.1186/

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Data deposition and availability

This thesis generated RNA sequenced data from a rare disease population (PMM2-CDG) which individual-level data was submitted at the database of Genotypes and Phenotypes (dbGaP) and are available for download by authorized investigators. Public summary-level phenotype data are available at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs003313.v1.p1.

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2-FF	2-fluorofucose
AR	Androgen receptor
APC	Antigen presenting cells
BC	Breast cancer
CASP8	Caspase 8
CCL	C-C Motif chemokine ligand
CDG	Congenital disorders of glycosylation
cIAP	Cellular inhibitor of apoptosis protein
CK	Cytokeratin
COA	Clinical Outcomes Assessment
ConA	Concanavalin A
CT	Cycle threshold
CTCF	Corrected total cell fluorescence
CXCL	C-X-C motif chemokine ligand
DMEM	Dulbecco's Modified Eagle Medium
DEG	Differentially expressed genes
DFS	Disease-free survival
E-SL	E-selectin ligand
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2

FADD	FAS-associated death domain
FBS	Foetal bovine serum
FC	Fold change
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FUT	Fucosyltransferases
GAGs	Glycosaminoglycans
G-CSF	Granulocyte colony-stimulating factor
Gal	Galactose
GalNAc	N-acetylgalactosamine
GDP	Guanosine diphosphate
Glc	Glucose
GlcNAc	N-acetylglucosamine
GNL	Galanthus nivalis
GO	Gene ontology
GPI	Glycosylphosphatidylinositol
GSEA	Gene set enrichment analysis
HCP	Healthcare professionals
HD	Hemidesmosomes
HER2	Human epidermal growth factor receptor 2
(Hr)QoL	(Health-related) Quality of Life
IEF	Isoelectrofocusing
Ig	Immunoglobulin
IL	Interleukin
IMD	Inherited metabolic diseases
IV	Intravenous
JNK	JUN N-terminal kinase
MAb	Monoclonal antibody
Man	Mannose

MAPK(KK)	Mitogen-activated protein kinase (kinase kinase)
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa B
NK	Natural killer
NS/NStim	Non-stimulated
ObsROM	Observer-Reported Outcomes Measure
PC	Principal component
PMM2	Phosphomannomutase 2
PPAIN	Professionals and Patients Associations International Network
PI	Public involvement
PRO(M)	Patient-reported outcome (measure)
PRRs	Pathogen recognition receptors
RD	Rare disease
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
sLe$^{X/A}$	Sialyl Lewis X/A
TAB	TAK1-binding protein
TACE	TNF-α converting enzyme
TAK1	Transforming growth factor b-activated kinase 1
TCGA	The Cancer Genome Atlas
Tf	Transferrin
TGF	Transforming growth factor
TLR	Toll-like receptor
TNBC	Triple negative breast cancer
TNF(R)	Tumour necrosis factor (receptor)
TRADD	TNF receptor type 1-associated death domain
TRAF	TNF receptor associated factor
TRECs	Undetectable T cells receptor excision circles
UDP	Uridine diphosphate

WT	Wild type
XMEN	X-linked MAGT1 deficiency with increased susceptibility to EBV-infection and N-linked glycosylation

CHAPTER 1.

MOTIVATION, AIMS AND OUTLINE

1.1. Motivation and aims

Glycosylation is an essential post-translational modification that occurs ubiquitously in the human body [1]. In addition to the genome or proteome, the repertoire of glycans through the organism - the glycome - represents an additional layer of critical information for all biological and physiological body functions [2]. The disruption of properly functioning glycosylation mechanisms harbours significant repercussions to the homeostasis. Ultimately, abnormal glycosylation leads to disease, whether the defects arise from inherited genetic pathogenic variants or are acquired, as is the case of congenital disorders of glycosylation (CDG) or cancer, respectively [3].

This thesis will focus on the mechanisms of two glycosylation disorders, namely the ultra-rare (but most frequent) CDG, phosphomannomutase 2 (PMM2)-CDG and the low incident triple negative breast cancer (TNBC). The study of these less frequent human glycosylation disorders is, on the one hand, critical for the understanding of their pathogenesis and the development of novel therapies which align with the Goal 3 of the 2030 Agenda for Sustainable Development of the United Nations [4] - "*Ensure healthy lives and promote well-being for all at all ages*" - and, on the other hand, informative and beneficial to other more common diseases that have been linked to alterations in glycosylation.

In the words of Dr. James K. Stoller, orphan and less common diseases "*may lack support and advocacy just as orphans lack parents*" [5]. Due to low disease prevalence, it is not only difficult for the patients to find often-limited experts and timely diagnosis, but also challenging for clinicians that disease awareness and standard-of-care guidelines are scarce. Furthermore, disease expertise is normally concentrated in very few, geographically dispersed clinical centres, which require long and costly travels, which in addition to treatment or vast symptom-management therapies, constitutes a huge financial burden [5], [6]. When it comes to conducting basic and

clinical research, investigators struggle to assemble suitable cohorts, to find appropriate models and other necessary resources as well as to find and hold enough funding opportunities [5]. In such a complex and demanding clinical setting, the concept of patient-centricity arises as an opportunity to conduct research right, timely and meaningfully by people with complex diseases.

Patient- or people-centricity is defined as "*putting the patient* (or person) *first in an open and sustained engagement of the patient to respectfully and compassionately achieve the best experience and outcome for that person and their family*" [7]. This is a culture that outdates the traditional, doctor- and disease-driven method and favours a modern approach that ensures that the patient needs and preferences are prioritized, not only in their care, but even in basic and clinical research. Besides evidently relevant in healthcare, people-centricity is gaining momentum in clinical trials and research, ensuring that research priorities, designs and outcomes are meaningful to the community [8].

Based on this new paradigm, the CDG & Allies - Professionals and Patients Associations International Network (PPAIN) was created in 2016. This is a community-led infrastructure composed of a broad network of scientists, healthcare professionals (HCP), families, and patient advocate groups with the goal of boosting accurate and timely diagnosis and promoting collaborative research, best practices, awareness, and education for CDG and other glycosylation-related disorders. Since its inception, the network activities focus on help mitigating the unmet needs of the community using a holistic approach, with the utmost priority of putting the patient first and in the centre of decision-making. To achieve this, CDG & Allies - PPAIN developed and systemically applies its own public involvement (PI) framework (Figure 1.1), conforming to existing guidance from funding bodies and authorities [9], [10]. Starting with the identification of unmet needs and pinpoint of research priorities to its dissemination and evaluation, patients, their families, and all other relevant stakeholders are recognized as partners in all stages with relevant roles that shape and drive the projects. Several examples using various methods can be highlighted to illustrate the framework. By applying quantitative, qualitative and mixed research methods, namely by using e-surveys and think tanks targeting families, researchers and HCP, the network longitudinally identified the unmet needs and potential solutions of the CDG community in a collaborative way [11]–[13]. Specifically, among others, a main concern was the limited, scattered, and inaccessible clinical information which propelled a series of literature reviews, gathering all available information on clinically relevant topics [14]–[25]. At the stage of designing and undertaking research, CDG & Allies - PPAIN's PI framework is well demonstrated by the methodological case study of the of the immune-related e-questionnaire for CDG – ImmunoCDGQ [26]. The inclusion of the family and professional advisory committees allowed on the one hand, an appropriate, targeted, and clear questionnaire structure and, on the other hand, guaranteed a successful questionnaire outreach, participation, and data collection [26]. Considering research analysis, families and external experts have been proven to be essential to

interpret results correctly due to their real-world and clinical experience [27]. Finally, when it comes to dissemination, implementation and evaluation, people involved in the projects ensure that the findings are widely disseminated, that they exert influence and drive changes for the improvement of health or care and that their impact is well understood and transmitted. A good example of this was the joint international multi-stakeholder effort for the development of PMM2-CDG clinical guidelines followed by their adaptation to a plain-language version [16], [28].

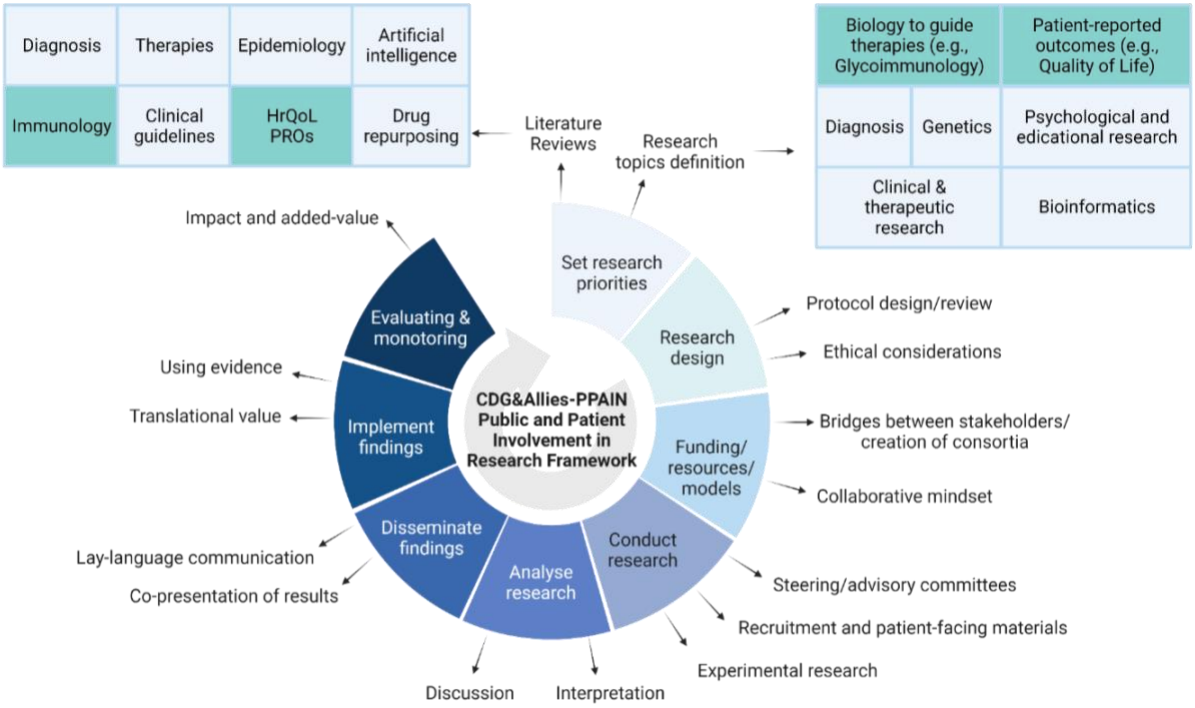

Figure 1.1. CDG & Allies - PPAIN public involvement in research framework. The framework is divided in 8 stages and the goals and roles that patient and the public play are described per stage. The framework shows an illustration of several PI strategies in line with the scope of this thesis, but it is not an exhaustive list of all the activities of the network. The topics for research and literature reviews highlighted in green represent the identified priorities that this thesis focus on.

This thesis was motivated by the CDG & Allies - PPAIN community-driven identification of gaps and research priorities in the two glycosylation diseases selected. Specifically:

- For PMM2-CDG, the studies described in this dissertation arise from (1) the lack of (or access to) samples associated with the rarity of the disease and the need for appropriate disease models to study the disease pathology [12]; (2) the necessity to investigate the impact of abnormal glycosylation in the immune system since this area was noted as highly unexplored and neglected following a literature revision [14]; and (3) the fact that impaired quality of life was identified as a major issue due to the complex and debilitating nature of the disease as well as its psychological,

financial and time burden [11], [29] and that there are no single tools that can measure patient-reported quality of life in a disease-specific manner [25].

- In the case of TNBC, the utmost patient-reported priority is the inexistence of a curative or effective treatment [30]. However, the current standards-of-care are limited to non-specific interventions [31], [32], which not only lack effectiveness, but also have significant side effects [33]. Unsurprisingly, treatment decisions have been shown to instigate patient distress [34]. One of the major gaps for therapy development for TNBC is its high heterogeneity [35], which will be addressed in this thesis.

Based on the abovementioned identified needs, and keeping in mind its people-centric relevance, the aims of this thesis were:

1. To identify, characterise and validate fibroblasts as a cellular model that facilitates CDG research, particularly, for the study of immunological mechanisms;
2. To create new insights into the immunopathology of PMM2-CDG, specifically focusing on the response to inflammatory insults and faulty inflammatory molecular mechanisms using comprehensive approach and potentially highlight new immunotherapeutic targets. This included transcriptomics, signalling pathways and *in vitro* validation whose data not only enlighten immune-related defects in PMM2-CDG but also aided in establishing possible interconnections with other signs and symptoms;
3. To identify and analyse existing health-related quality of life (HrQoL) patient reported outcomes (PRO) specific for the signs and symptoms that mostly affect the daily lives of CDG patients, according to the community. Secondarily, we aimed to provide the scientific community with alternative tools that can not only aid the establishment of the disease natural history but also aid future therapy approval (for instance, immunotherapies);
4. To study and validate a glycosylation-related biomarker that can help clarify TNBC subtypes and explain its heterogeneity to contribute to TNBC stratification which might benefit the development of more targeted therapeutic options.

All in all, this thesis combines a series of contributions that fall into different stages of the research and development (R&D) process, from pre-clinical to clinical research outcomes research, showing examples of how research can be inspired by real-word evidence and demonstrates the people-centric approach as the driving force for scientific and therapeutic innovation for complex diseases.

1.2. Thesis outline

This thesis is structured in 7 chapters and an additional section with supplementary materials (Figure 1.2). While Chapter 1 and 2 are introductory sections, Chapter 3 to 6 contain the investigational work conducted, finishing with Chapter 7 as the concluding chapter. In detail:

- Chapter 1 contains the description of the motivations, aims and structure of the thesis.
- Chapter 2 offers the reader a general introduction to provide context on the topics addressed in this thesis. It starts with an overview of the glycosylation process and its roles in biological processes, health, and disease, followed by a summary of some genetic, biochemical, cellular, and clinical features of the two glycosylation disorders, namely CDG and cancer. After that, this chapter provides an overview of the immune system and how the glycosylation can modulate its function. It proceeds with the immune-related aspects of the abovementioned disorders, including immunological hallmarks, presentations, mechanisms, and therapeutic approaches, with emphasis on the disorders under study - PMM2-CDG and TNBC. Then, the concept of patient-centricity is introduced along with its relevance and potential in biomedical and clinical research. In line with this topic, PI strategies across the R&D process are described. Lastly, this chapter explains the significance of collecting patient reported data with particular focus on HrQoL as an outcome.
- Chapter 3 describes the studies performed to validate skin fibroblasts as an adequate model to investigate immunopathology in rare genetic disease, considering the challenge of lack of access to patient samples and models, highlighted by the community. By using a transcriptomics approach plus several validation assays, we show that the fibroblasts' response can resemble immune cells' response when challenged with a pro-inflammatory stimulus. Furthermore, we deeply characterize the molecular response of fibroblasts to tumour necrosis factor (TNF)-α and predict cell-cell interactions based on the genetic alterations. With this chapter, we provide evidence that suggest the use of patient-derived fibroblasts stored in cell repositories and biobanks for immunological studies when other alternative models are not available.
- Chapter 4 takes the learnings from the previous chapter and resorts to patient-derived fibroblasts to study the molecular mechanisms behind immunological involvement observed in PMM2-CDG. Following the assessment of their glycophenotypes, several gene expression differences are identified between the PMM2-CDG and control groups when stimulated with the inflammatory TNF-α

stimulus. These differences translated in several pathways and biological processes impaired in PMM2-CDG which were then validated using several experimental techniques. In the end, this work increases the knowledge on the immunopathology of PMM2-CDG and provides potential immune-related therapeutic targets.

- Chapter 5 focus on the people-centred methodology applied for the identification of PRO targeting HrQoL for PMM2-CDG. By including multi-stakeholders and using both quantitative and qualitative methods, a tailored strategy was devised to identify existent HrQoL questionnaires targeting the most impactful signs and symptoms which include immune-related issues. The work performed in this chapter is a contribution to answer a huge unmet need: the inexistence of PMM2-CDG-specific HrQoL tools which can not only help defining the real burden of the disease but also constitute a clinical outcome to aid future therapy approval.
- Chapter 6 presents the experimental work developed towards the understanding of how glycosylation underlies TNBC heterogeneity, a main hurdle behind the lack of effective treatment and thus contributing to low patient's quality of life. Specifically, the characteristics of the TNBC cohort included were analysed depending to the expression of the glycan-structure $sLe^{X/A}$ and the findings validated resorting to public gene expression data. Based on these characteristics and signalling alterations, $sLe^{X/A}$ might aid TNBC screening and a mechanistic hypothesis on the role of this glycan is provided.
- In Chapter 7, a discussion, main conclusion, and future perspectives of the results of this thesis are provided.
- The supplementary material contains all relevant additional information to the main chapters.

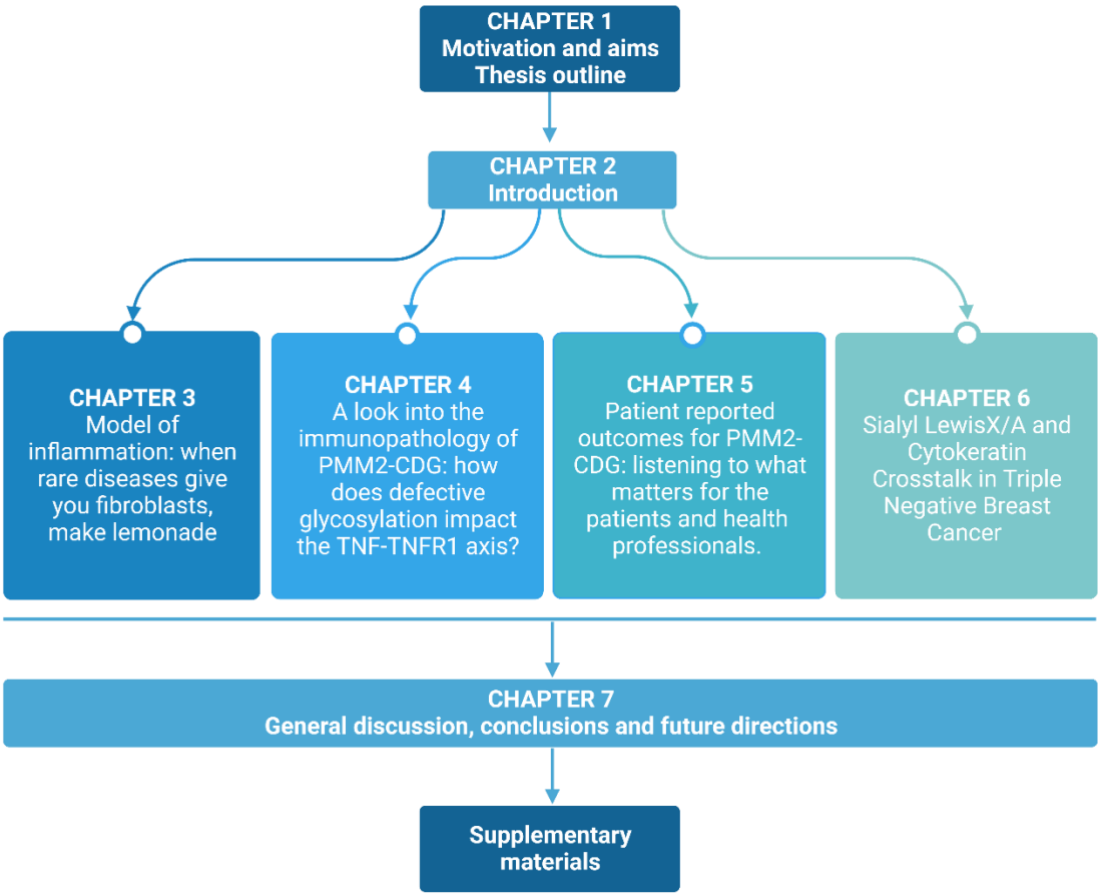

Figure 1.2. Outline and organization of the present thesis.

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CHAPTER 2.

INTRODUCTION

The content of this chapter contains parts of the following publications:

Francisco R, Brasil S, Poejo J, Jaeken J, Pascoal C, Videira PA, dos Reis Ferreira V. Congenital Disorders of Glycosylation (CDG): state of the art in 2022. (Accepted by the Orphanet J Rare Dis.)

Pascoal C, Francisco R, Ferro T, Dos Reis Ferreira V, Jaeken J, Videira PA. 2019. CDG and immune response: From bedside to bench and back. J Inherit Metab Dis. doi: 10.1002/jimd.12126.

Altassan R, Péanne R, Jaeken J, Barone R, Bidet M, Borgel D, Brasil S, Cassiman D, Cechova A, Coman D, Corral J, Correia J, de la Morena-Barrio ME, de Lonlay P, Dos Reis V, Ferreira CR, Fiumara A, Francisco R, Freeze H, Funke S, Gardeitchik T, Gert M, Girad M, Giros M, Grüne-wald S, Hernández-Caselles T, Honzik T, Hutter M, Krasnewich D, Lam C, Lee J, Lefeber D, Marques-de-Silva D, Martinez AF, Moravej H, Õunap K, Pascoal C, Pascreau T, Patterson M, Quelhas D, Raymond K, Sarkhail P, Schiff M, Seroczyńska M, Serrano M, Seta N, Sykut-Cegielska J, Thiel C, Tort F, Vals MA, Videira P, Witters P, Zeevaert R, Morava E. International clinical guidelines for the management of phosphomannomutase 2-congenital disorders of glycosylation: Diagnosis, treatment and follow up. J Inherit Metab Dis. 2019 Jan;42(1):5-28. doi: 10.1002/jimd.12024. Review. Erratum in: J Inherit Metab Dis. 2019 May;42(3):577.

Pascoal C, Brasil S, Francisco R, Marques-da-Silva D, Rafalko A, Jaeken J, Videira PA, Barros L, dos Reis Ferreira V, Patient and observer reported outcome measures to evaluate health-related quality of life in inherited metabolic diseases: a scoping review, Orphanet J Rare Dis. 2018 Nov,13:215. doi: 10.1186/

2.1. Glycosylation: a scale's arm between health and pathology

Living organisms are deeply structured and organized entities characterized by their ability to breathe, grow, move, reproduce, and sense changes in their environment [1]. From unicellular to multicellular organisms, these properties rely on several pieces of information stored within or on the cells which, all together, instruct fine-tuned processes that lead to a perfectly functional organism [2].

There are three chains that support life which, when disturbed, compromise the healthy state of an organism. Glycans have been referred to as the third chain of life, after nucleic acids and proteins, being central to many biological mechanisms [3]. The glycome, which refers to all sugars of an organism whether free or present in more complex molecules, constitutes the biological code with the greatest information diversity that populates an organism [4]. In fact, just like pathogenic genetic variants and protein dysfunction lead to several disorders, glycan and glycosylation abnormalities have been linked to several human diseases or conditions [5]. The sugar code has been considered the third alphabet of life [6].

2.1.1. Glycosylation: a ubiquitous and essential post-translation modification

Glycosylation is the assembly, processing, and addition of sugars (glycans) to proteins, lipids and, more recently pinpointed, small RNAs [7], [8]. It is one of the most ubiquitous, complex, and essential post-translational modifications, with various critical biological and physiological functions. Glycans typically decorate the cell surface and secreted proteins as well as some cytosolic proteins. Indeed, at least 50 % of the human proteome is estimated to be glycosylated [9]. Structurally, glycans are relevant since they often comprise a significant portion of glycoproteins [10]. Function-wise, glycans ensure protein maturation and stability, functional modulation (e.g., immunogenicity), or even confer new functions to proteins (e.g., through glycan recognition by lectins), among others [11]–[14].

The glycosylation machinery comprises a set of proteins that process, transport, and assemble monosaccharides derived from the primary metabolism into linear or branched glycan chains [7]. The resulting glycoconjugates are highly diverse, which stems from the glycosylation type, the nature and combination of the constituting sugars, as well as from the glycosidic bonds

involved. Unsurprisingly, most proteins have several glycoforms that can be expressed within the same cell or be cell/tissue/organ-specific. This diversity has multiple implications in protein function, cellular interaction/communication and, ultimately, in organ homeostasis [15]–[17].

The most well described mammalian protein glycosylation pathways are N-linked glycosylation and O-linked glycosylation. N-glycosylation is the most extensively studied pathway and takes place in an ordered, multistep process in different cellular compartments. It is initiated in the cytoplasm with the formation of activated sugars (e.g., GDP-mannose (Man) and UDP-glucose (Glc)) and the synthesis of dolichol-linked precursor oligosaccharides in the endoplasmic reticulum (ER) membrane. In the ER lumen, the precursor oligosaccharide assembled onto the lipid carrier, is transferred to an asparagine residue of a nascent protein by an oligosaccharyltransferase. The terminal N-glycosylation step concerns the trimming and processing of the glycans and takes place both in the ER and Golgi complex. The inter-organellar transport system as well as substrate availability and glycosyltransferases and glycosidases specificities are responsible for the generation of a wide array of glycans with increasing degrees of branching and complexity [7]. In contrast with N-glycans, O-glycans are built by monosaccharide transfers catalysed by glycosyltransferases mainly located in the Golgi complex. The initial step of the most prevalent type of O-glycosylation occurs in the rough ER or the cis Golgi with the transfer of N-acetylgalactosamine (GalNAc) to the hydroxyl group of a serine or threonine residue of the protein. This core structure can then be processed to generate different mucin-type core structures, which may be further elongated and modified. Besides GalNAc-O-glycans, other O-glycans containing either fucose or xylose as the first monosaccharide may also be synthetized [7]. Glycosaminoglycans (GAGs) are negatively-charged, long, unbranched molecules containing repetitive disaccharide units, predominantly found at the extracellular matrix (ECM) and cell membranes. The variation and differential arrangement of these monosaccharides gives rise to different types of GAGs (e.g., heparin and heparan sulfate) [7]. When GAGs are linked to proteins, they originate proteoglycans [7]. Glycosylphosphatidylinositol (GPI) anchors are glycan structures that attach proteins to the outer layer of the cytoplasmatic membrane. These anchors have three components: a phosphatidylinositol lipid, a glycan core, and a phosphoethanolamine linker that connects to the protein. GPI anchors are widespread among eukaryotes and have various functions, such as acting as receptors, transporters, and adhesion molecules [18].

Lipid glycosylation gives raise to glycosphingolipids which reside in the cell membrane. Glycosphingolipids are formed by a ceramide backbone covalently linked to a glycan moiety. They are synthesized by the sequential addition of sugar residues to ceramide by glycosyltransferases in the ER and the Golgi compartment. First, the addition of UDP-Glc or UDP-galactose (Gal) to ceramide give rise to glucosylceramide or galactosylceramide respectively. Then, they can be

further modified by different glycosyltransferases to generate various glycosphingolipids [19]. Figure 2.1 illustrates these different types of glycosylation pathways as well as their products.

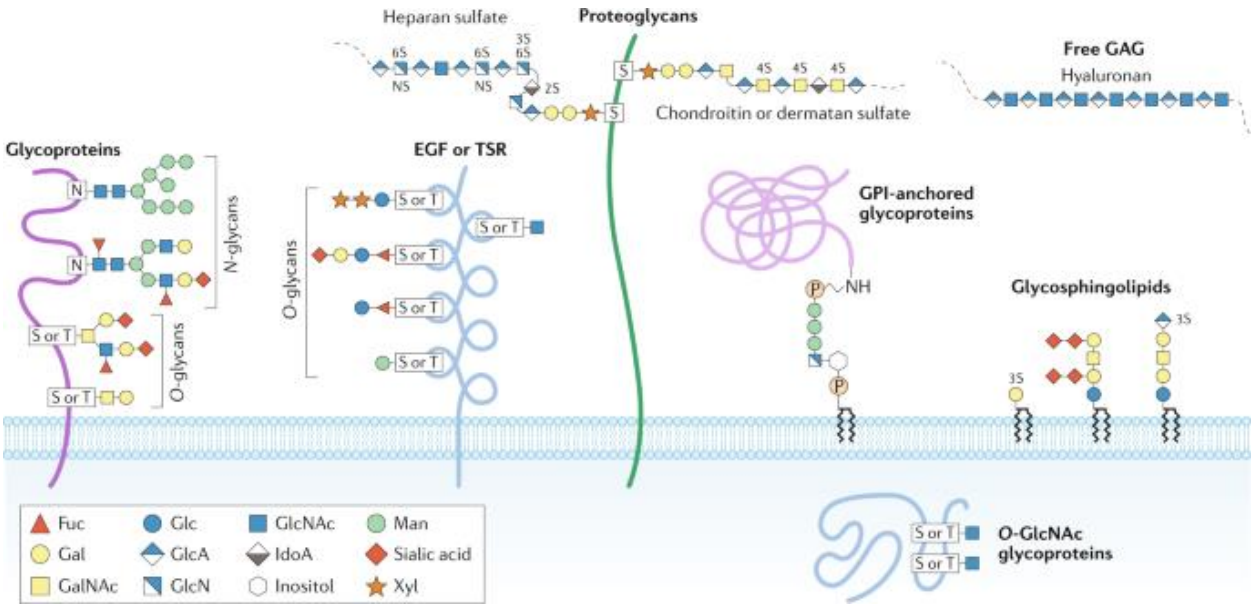

Figure 2.1. Main mammalian glycosylation pathways. Proteins and lipids are modified with glycan moieties by covalent linkages. The type of glycosylation is classified according to both the conjugate, the linkage, and the attached carbohydrate. Adapted from [5].

2.1.2. Human Disorders of Glycosylation

The dysregulation of glycosylation pathways causes alterations in the human glycoprofile which, by affecting crucial physiological processes, are the basis of or contribute to the pathology of many diseases [5]. Different specific changes in glycan composition are directly linked to different phenotypes, depending on their importance in development stages, age-dependent and tissue expression [20]–[22]. Accordingly, a defect in a sole glycosylation-related enzyme can have widespread consequences in the organism causing multi-system disease just as induced and local glycosylation changes can cause site-specific pathologies. This is, for example, the case of most congenital disorders of glycosylation (CDG) and inflammatory bowel disease (IBD), respectively [23], [24]. Nevertheless, in the middle of the spectrum, many other diseases have been associated with glycosylation alterations, like cancer, autoimmune and other neurological diseases [16], [25], [26].

In the scope of this thesis, the next sections will further introduce the clinical picture of CDG and cancer as well as their glycosylation characteristics.

2.1.2.1. Congenital Disorders of Glycosylation

CDG are a group of inherited metabolic diseases (IMD) originated by mutations in genes involved in glycan biosynthesis. Over the years, the number of CDG has been growing and so far, 163 CDG genetic defects are known encompassing 217 different diseases (Figure 2.2). They have been classified according to the affected glycosylation pathways, namely N-glycosylation, O-glycosylation, GPI-anchor synthesis, lipid glycosylation, and multiple and other glycosylation pathways (Supplementary Table 2.1).

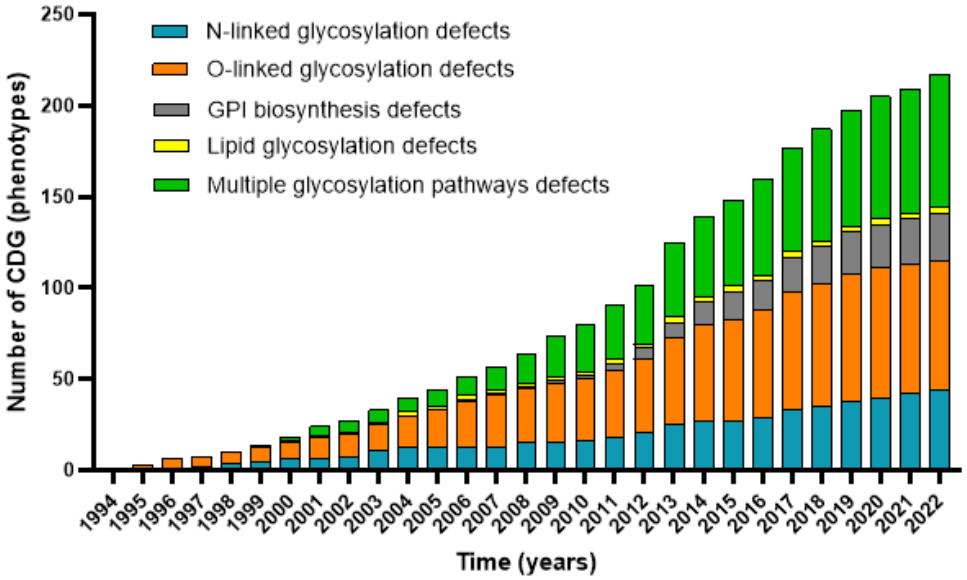

Figure 2.2. Graphical representation of the yearly distribution (from 1994 to 2022) of reported CDG phenotypes according to the underlying affected glycosylation pathway(s). The years correspond to when the association between the gene and the phenotype was established.

CDG genetic transmission is usually autosomal recessive, but seven and six percent of the clinical presentations have an autosomal dominant and an X-linked transmission, respectively (Supplementary File 2.1) [27]–[29]. Only one CDG - XYLT1-CDG - has been reported to have epigenetic inheritance [30]. Contrary to other IMD families, they are due to defects occurring in several cell organelles, mainly the cytosol, the ER, the ER-Golgi intermediate compartment, the Golgi, and the sarcolemmal membrane [31]. The defects are associated with glycoprotein and glycolipid glycan assembly and remodelling. While type I CDG are caused by abnormalities in the formation of the oligosaccharide structure on the glycolipid precursor before the attachment to the asparigine residue of a protein, type II CDG involve defects in the control of the N-linked branching structure on the nascent glycoprotein [32]. These defects cause various symptoms that can manifest from birth or even the ante-natal period [33].

The heterogeneity of CDG is striking from several points of view. The large majority (~88 %) are multisystem diseases [34]. The most typical CDG symptoms are associated with neurological and developmental disabilities [35]. However, their multisystem nature also causes serious hepatic, gastrointestinal and immunological problems that require close and continuous healthcare [36]–[38]. The mono-system diseases (~12 %) affect either the brain, the eyes, the skin, the skeleton, the skeletal muscles, the liver, the red blood cells, or the neutrophils [34], [39], [40]. The severity of CDG is also highly variable with their clinical expression extending from prenatal death (and probably even miscarriage) to mild adult involvement [41]. The heterogeneity is even more pronounced since a gene defect can result in multiple clinical presentations or have different inheritance patterns depending on the involved variant [42]–[44]. Furthermore, phenotypic diversity and severity can be affected by the variant type and location within the gene (with more severe phenotypes being associated with greater enzymatic disruption [45]–[47]) and by other modifiers, including additional defective glycogenes and recombinant mitosis [48], [49]. All these factors pose a challenge for diagnosing CDG.

Since there is no general diagnosis method for all CDG, the diagnosis of these diseases still counts on the classical and initial screening test - the serum transferrin (Tf) isoelectrofocusing (IEF) (Figure 2.3). This test relies on identification of the migration pattern of the Tf protein depending on its degree of sialylation. Briefly, the resulting pattern can show a complete or incomplete lack of glycans (type I pattern) indicating N-glycosylation defects in the early steps of N-glycosylation or show the presence of truncated glycans (type II pattern) suggestive of glycan processing defects in the Golgi [50]. Due to the nature and complexity of the disorders, around 50% of CDG cannot be detected by Tf IEF or show inconsistent results (Supplementary File 2.1). The second screening CDG test is the serum apolipoprotein C-III IEF, but this is abnormal in a very small number of CDG, namely the core 1 mucin type O-glycosylation disorders. This test is usually performed after finding a type II serum Tf IEF pattern to confirm a combined N- and O-glycosylation defect. When Tf IEF shows a normal pattern and with strong CDG clinical suspicion, other routine and specialized biomarker analyses can be performed (Supplementary File 2.1, Figure 2.3). Nevertheless, and due to all the limitations, CDG gene panels and whole exome/genome sequencing have become widely used diagnostic tools (Figure 2.3).

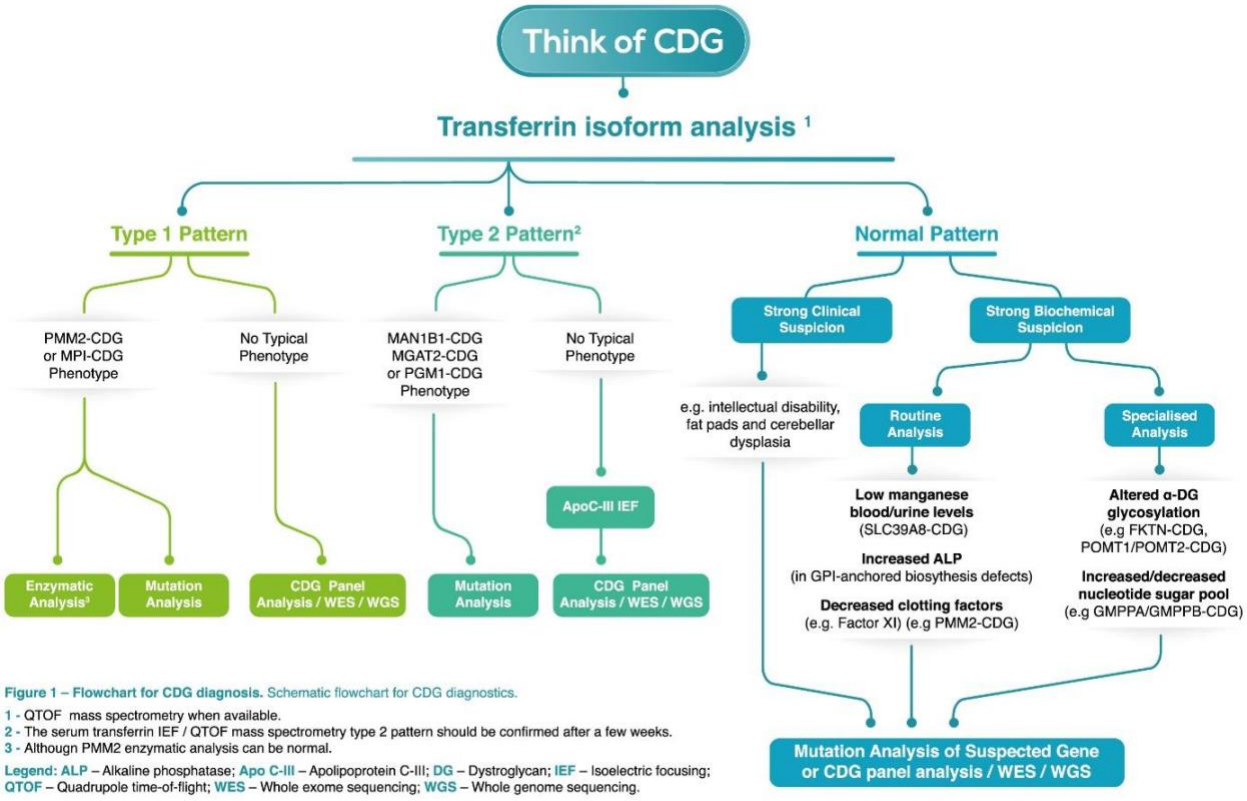

Figure 2.3. Diagnostic decision tree of CDG diagnosis following transferrin isoelectric focusing (Tf IEF) analysis. Recognizable phenotypes and some biomarker can aid in the diagnostic of CDG, nevertheless, needs to be confirmed by genetic testing, whether it is targeted gene analysis, a CDG gene panel or next generation sequencing techniques.

Despite the genomic diagnosis revolution, the discovery of several biomarkers and the development of several disease models for CDG, very few targeted therapies exist for this group of disorders, and most available therapies are restricted to symptom management [51], [52]. The treatments (Supplementary File 2.1) include not only surgical approaches (e.g., liver and heart transplantation), gene therapy, clinical trials in different phases, and dietary supplementation (some being administered under compassionate and off-label use programs) but also other therapies in earlier phases of testing (i.e., *in vitro* and *in vivo* studies). Notably, until 2022, most of these treatments have not been approved by regulatory bodies or are available in the market [53]. Some therapeutic opportunities currently being investigated include dietary sugar and trace element (e.g., vitamin) supplementation, drug repurposing, and gene replacement strategies [51], [52], [54]. Unfortunately, until 2022, drug development is under investigation for only 59 CDG among all 217 discovered so far (Supplementary File 2.1). Effective and targeted therapeutics available are Man supplementation and liver transplantation for MPI-CDG (MIM: 602579), heart transplantation in DOLK-CDG (MIM: 610768) and Gal supplementation in PGM1-CDG (MIM: 614921) (Supplementary File 2.1) [51], [52].

2.1.2.2. Cancer

Cancer is a collective term that encompass diseases characterized by an uncontrolled division of abnormal cells in the organism. The development, sustainment and spreading of cancer is enabled by a set of hallmarks and enabling characteristics. There are currently 8 hallmarks of cancer, namely sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing, or accessing vasculature, resisting cell death, deregulating cellular metabolism and avoiding immune destruction. Additionally, two enabling characteristics contribute to the acquisition of the cancer hallmarks, particularly, a tumour-promoting inflammation and genome instability and mutation (Figure 2.4) [55], [56]. More recently, two additional emerging hallmarks (unlocking phenotypic plasticity and senescent cells) and enabling characteristics (nonmutational epigenetic reprogramming and polymorphic microbiomes) have been proposed [56].

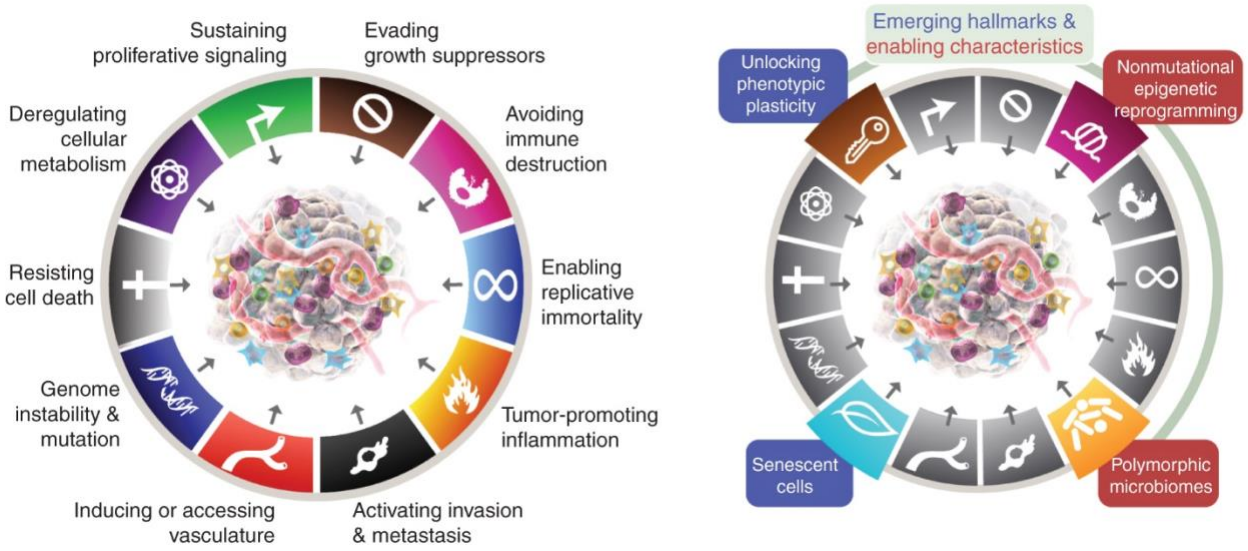

Figure 2.4. The hallmarks of cancer. Currently, there are 8 hallmarks and 2 enabling characteristics of cancer. Additionally, two other hallmarks and enabling features seem to be emerging. All these characteristics are shown in the illustration. Image adapted from [56].

Over the years, it has been made clear that glycosylation plays an important role in the mechanisms of all hallmarks of cancer [57]. During carcinogenesis and tumour progression, the glycosylation pattern of cell surface and glycoproteins is remodelled acquiring profiles that aid their malignancy. The aberrant glycosylation profiles consist of under- or overexpression of glycan structures as well as the emergence of novel and truncated structure and are thought to be due to genetic and epigenetic alterations as well to folding, mislocalisation and expression changes of

glycosyltransferases and their substrate availability [57], [58]. These fundamental glycosylation changes during malignant transformation give rise to glycan epitopes that are known to have specific roles in the cancer characteristics [58]. Both patients' clinical presentation, diagnosis and treatment are highly dependent on the cancer type, stage, and its body location. Nevertheless, glycan-based tumour-associated antigens have been proven to have implications both for cancer diagnosis and targeted-treatment [59], [60].

2.2. The immune system: a concerted surveillance ally

The human body is a complex and structured organism made up of specialized cells and extracellular materials which are organized in tissues, organs, and body systems. In a finely balanced and regulated motion, all systems are interconnected and depend on each other to maintain homeostasis. Fundamentally, the immune system contributes to the optimal living state by protecting the body against pathogens, foreign agents and unhealthy infected or transformed cells.

The immune system function relies on three main concepts: recognition, elimination, and memory. Immune cells are constantly surveilling the health status of the body by recognizing and distinguishing between self (normal) components from non-self (foreign) entities. The recognition of external or altered threats activates controlled mechanisms of elimination to restore a natural state. Luckily, some immune system cells can generate specific molecules that can recognize previously eliminated insults more effectively. These interlocked mechanisms of identification, clearance and memory are led by both the innate and acquired immune responses.

2.2.1. Innate immune system

Innate immunity constitutes the earliest protection barrier that protects the body against foreign organisms and non-self structures [61]. Innate physical barriers include the skin and the mucous membranes and are associated with chemical barriers like unsuitable pH levels for microorganism entry [62]. Several soluble molecules (e.g., cytokines ang complement proteins) and immune cells, namely natural killer (NK) cells and phagocytes, including, neutrophils, eosinophils, monocytes, macrophages, and dendritic cells, are physiological players of innate immunity (Figure 2.5). Though pathogen recognition receptors (PRRs), these cells respond to pathogen-associated molecular patterns that initiate phagocytosis, mediate direct cytotoxicity, and eliminate extracellular microbes [63]. Besides, these receptors can also recognize metabolic products related to tissue damage also known as damage-associated molecular patterns, activating an inflammatory response that promotes tissue regeneration [64].

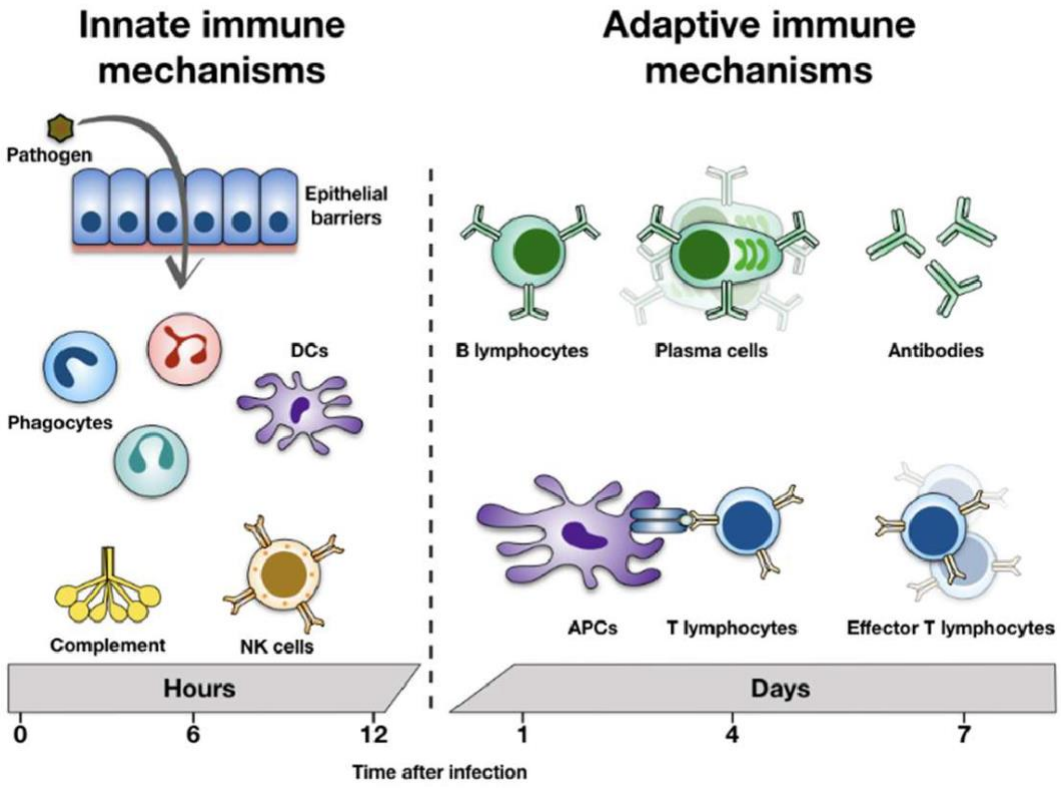

Figure 2.5. Innate and adaptive immune mechanisms and their activation across time. Graphic representation of some innate and adaptive immune response mechanisms and illustrative examples of the influence of glycans in the function and recognition by immune cell receptors and other signalling molecules.

The rapid response of the innate immune players can lead to the complete clearance of pathogens and other insults. However, when this response is insufficient, recruiting mechanisms take place to activate antigen specific B and T lymphocytes, eliciting an adaptive immune response (Figure 2.5).

2.2.2. Adaptive immune system

The adaptive response includes B and T cells, whose surface receptors have high specificity for the antigen and are clonally distributed on individual cells. Importantly, since some clonally expanded B and T cells differentiate into memory cells, the adaptive immunity offers long-lasting protection through immunological memory [65], [66]. While B cells can directly recognize many different soluble antigens and mediate humoral adaptive immunity, T cells can only recognize them if they are presented by antigen presenting cells (APC) via the major histocompatibility complex (MHC) molecules and mediate cellular adaptive immunity [65].

Humoral adaptive immunity is also called antibody-mediated immunity. It relies on the capacity of B cell to produce and secret antigen-specific antibodies (or immunoglobulins - Ig) [67].

When these antibodies bind to their specific antigens, effector functions are set-up leading to neutralization, opsonization increasing phagocytosis efficacy, activation of the complement cascade and antibody dependent cytotoxicity mediated by NK cells [68]. Besides their role in Ig production, B cells also have the capacity to activate cellular adaptive immunity, by presenting antigens to T cells, like other APC. There are two classes of T cells which are activated by APC: helper T cells and cytotoxic T cells. Helper T cells can be of several subsets and are characterized by giving support and providing signals to other immune cells mainly by the secretion of cytokines. Depending on which subset they belong to, they are responsible for different effector functions [69]. By doing this, for instance, they aid Ig production by B cells differentiation, modulate macrophage phagocytic capacity and cytotoxic T cells expansion [69]. Cytotoxic T cells, as the name indicates, can directly recognition and kill target cells (i.e., viral-infected cells, intracellular pathogens, and tumour cells). Once activated, these cells suffer clonal expansion into effector cells and memory cells. While effector cytotoxic T cells mediate cell killing by inducing apoptosis though different pathways, memory cytotoxic T cells provide immunological memory, capacitating the immune system towards next events of infection or other challenges [69].

Beside a tightly regulated interaction between the immune components is mandatory for its proper functioning, other players can influence and support the immune system behaviour. Fibroblasts' role in influencing the immune system has been increasingly recognized. They can not only interact with several immune cells and recruit and activate immune cells through the expression and secretion of soluble mediator but also interact exert immunosuppressive roles [70], [71]. Just as immune cells' function is important for maintaining a correct physiological state, fibroblasts are equally important. In fact, dysregulation of fibroblasts' immune-related function have been associated with several inflammatory and immune diseases [72]–[74].

An efficient immune response demands a constant and complex molecular and cellular crosstalk between all players described and more, to detect pathogens, to distinguish self from non-self and to enhance the response to forthcoming infections. All interventions of the immune response are indispensable for the prevention and eradication of infections, as well as to prevent immune dysfunction. Nevertheless, not less important are the glycans that decorate the players of immune system, exerting vital modulatory roles on its function.

2.2.3. The significant role of glycosylation for the immune system

The immune system is a paradigm of glycosylation control and fine-tuning. Glycoimmunology is a relatively recent research field that has the ambitious aim of further unravelling and understanding the complexity of the glycan landscape in the immune system/response. The immune response relies upon countless cell-cell contacts, recognition of

self- and non-self antigens, with glycans being present in virtually all the constituents of the immune system. Hence, defects in glycosylation may have a differential impact on the immune status, resulting in a broad spectrum of clinical manifestations and outcomes [75].

Glycans, due to their ubiquitous expression in practically all cell surface and secreted proteins, add yet another layer of intricacy and diversity to the immune response. Both glycans and glycan-binding proteins create a platform of contact and communication between immune cells. Here, the pivotal and widespread role of glycosylation is made evident by the array of molecules of the immune system that are glycosylated. These include immunoglobulins, adhesion molecules (e.g., integrins and selectins), PRRs (e.g., Toll like receptors – TLR - and lectins) and complement molecules, cytokines, chemokines and (many of) their receptors [76]–[85].

The role of human glycans in the immune response is depicted in two interrelated scenarios: (1) all pathogens require contact with host glycans for infection; (2) glycans ornamenting molecules of the immune response influence their properties, the subsequent signalling pathways, and, consequently, the triggered immune response.

Pathogens can interact directly with host glycans through glycan-binding proteins (e.g., pili) expressed by the pathogens [86]. Glycans can also indirectly influence pathogen recognition by promoting or hindering ligand accessibility at the host cell surface [87], [88]. Table 2.1 includes examples of direct and indirect glycan recognition by pathogens. Interestingly, the glycan composition of the pathogen itself, can serve to escape immune surveillance, recognition, and elimination [89]. In some cases, microorganisms can even appropriate the host-glycan to escape immune system-mediated recognition and destruction [90]. In the context of human defects of glycosylation, the glycan dependence seems not only to render some infectious agents inability to infect but also to render resistance from host cells to particular pathogens [91], [92].

These findings can provide strategies to control infections by microorganisms that are unable to adapt to glycan-depleted cellular landscapes.

Table *2.1.* Host glycan recognition by pathogens to promote infection and evade the immune response.

Glycan recognition, mimicry, and evasion by pathogens			
Direct recognition			
Pathogen / disease	**Host glycan**	**Mechanism**	**Reference**
E. coli / urinary tract infection	Gal-β1,4-D-Gal	P pili mediated adhesion	[86]
E. coli K1 / meningitis	N-glycans on the Ecgp96 glycoprotein	Outer membrane protein A- mediated recognition	[93]
P. aeruginosa / respiratory tract infections	D-Gal L-fucose	Lec A lectin Lec B lectin Recognition and adhesion Epithelium permeability	[94], [95]

Influenza A virus/flu	Sialylated cell surface glycans	Hemagglutinin (host internalization) and neuraminidase (release)	[96]
Polyomavirus	Sialylated glycans, non-sialylated GAGs	Major capsid proteins / for internalization	[97]
	Indirect recognition		
Hepatitis B virus / hepatitis	Core-fucosylated N-glycans	Envelop proteins / facilitate endocytosis	[88]
S. aureus/ detrimental bacterial infection	O-glycosylated mucins	Protect epithelial cells / hinder pathogen access	[87], [98], [99]

Glycosylation of host immune receptors governs the proper initiation of the immune response. Since glycans usually constitute a significant portion of the whole receptor, they may affect how receptors fold and associate among themselves (or with other proteins), as well as their stability and half-life [100], [101]. Most PRRs, namely TLR, calcium-dependent C-type lectins (such as dectin-1 and DC-specific intercellular adhesion molecule-3-grabbing non-integrin - DC-SIGN) and complement-related proteins (e.g., Man-binding lectin) are functionally dependent on glycosylation. Glycans may also be ligands of human lectins such as selectins and siglecs, among others. These interactions act mostly by modulating leukocyte trafficking and other events of the immune response. Table 2.2 include examples of glycan interference in the function of specific immune receptors and other immune signalling molecules. Understanding the mechanism of how glycans modulate or fine tune the properties of immune receptors and other molecules widens our comprehension of physiological and pathological immune conditions.

Table *2.2*. Glycans affecting recognition by and function of immune cell receptors and other signalling molecules.

Molecules	Glycan(s)	Mechanism	Reference
TLR2	N-glycans	Biosynthesis and secretion of the receptor	[102]
TLR3 and TLR4	N-glycans	Receptor integrity and biological function	[103], [104]
TCR	N-glycans	TCR clustering and signalling	[105]
TCR	O-glycans (namely GlcNAc glycoproteome)	T-cell activation and influencing TCR interactions with external galectins	[106]
DC-SIGN	N-glycans	Receptor diffusion, and modulation of the binding strength to bacterial antigen	[107]
MHC I	Sialylated N-glycans	Cell surface expression (possible effect on protein turnover)	[108], [109]
MHC II	N-glycans	Recognition and binding of antigens to be presented to T cells	[110]
Immunoglobulins	Sialylated N-glycans	Recognition by FC receptors	[111]–[114]

Selectin ligands	Sialyl-Lewis X (sLe^X) and 6'-sulfo-sLe^X on N- and O-glycans	Recognition by L-, E- and P-selectins, leukocyte migration/recruitment	[115]–[119]
Siglecs	Glycans with terminal sialic acid	Self and foreign antigens discrimination and prevention of the overactivation of the immune system	[108], [120]–[123]

2.3. Immune aspects of disorders of glycosylation

Given the importance of glycosylation for the function of the immune system, it is not surprising that disorders of glycosylation present alterations of normal immunological processes causing immune dysfunction or dysregulation. On the one hand, the ultra-heterogeneity of CDG is well mirrored by their immunological presentation. The degree of CDG immunological dysfunction among and within each CDG ranges from major immunodeficiency phenotypes to recurrent infections without clear cellular and/or biochemical anomalies [38], [124], [125]. On the other hand, the analysis of the cancer hallmarks clearly show that malignant cells can evade immunological processes, take advantage of inflammatory environments, and acquire immunological features that aid in their progression and dissemination [55], [57]. The next sections will further elaborate on the immune aspects of CDG (with focus on CDG with major immunological involvement and on the most frequent CDG - PMM2-CDG) as well as of TNBC.

2.3.1. CDG with major immunological involvement

To date, twelve CDG have immunological dysfunction or manifestations as one of the main disease hallmarks. The analysis of the different presentations and impaired mechanisms among these CDG allowed their division in six different classification groups, according to the recently updated classification of the International Union of Immunological Societies (Figure 2.6) [126].

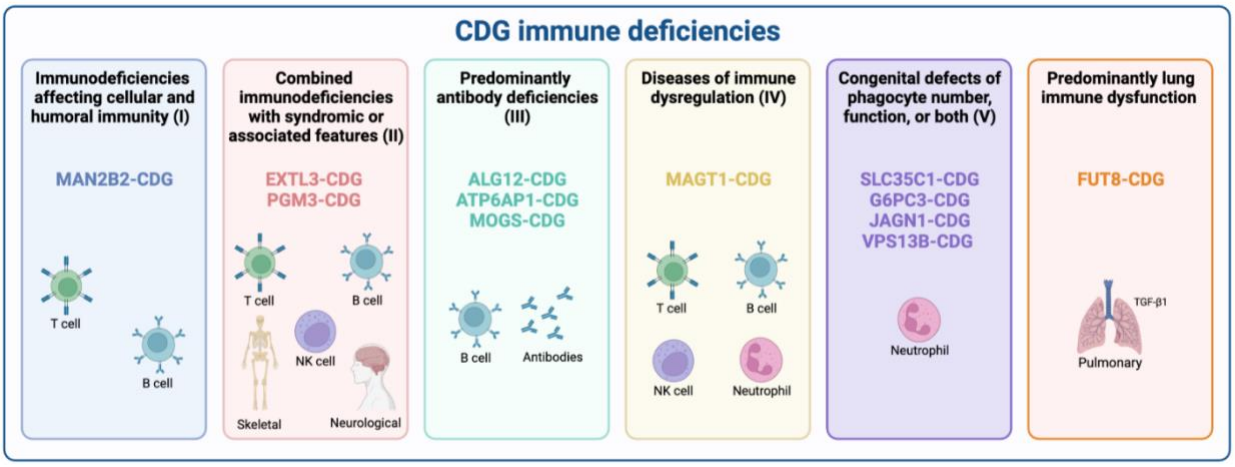

Figure 2.6. Classification of the CDG immune deficiencies according to their underlying immunological defects according to the updated IUIS classification [126]. CDG immune deficiencies fall under five different standard categories: (I) immunodeficiencies affecting cellular and humoral immunity (MAN2B2-CDG); (II) combined immunodeficiencies with syndromic or associated features (EXTL3- and PGM3-CDG); (III) predominantly antibody deficiencies (ALG12-, ATP6AP1- and MOGS-CDG); (IV) diseases of immune dysregulation (MAGT1-CDG); and (V) congenital defects of phagocyte number, function, or both (SLC35C1-, G6PC3-, JAGN1-, VPS13B -CDG). Existing evidence shows that FUT8-CDG is a disease with predominant lung immune dysfunction, but it is still not clear in which IUIS classification it falls in.

The inclusion of each CDG in these different groups will be detailed below. An overview of the affected enzymes in each one of the twelve CDG, impaired glycosylation pathways and subcellular localization is depicted in Figure 2.7 and their reported immunological manifestations are portrayed in Table 2.3.

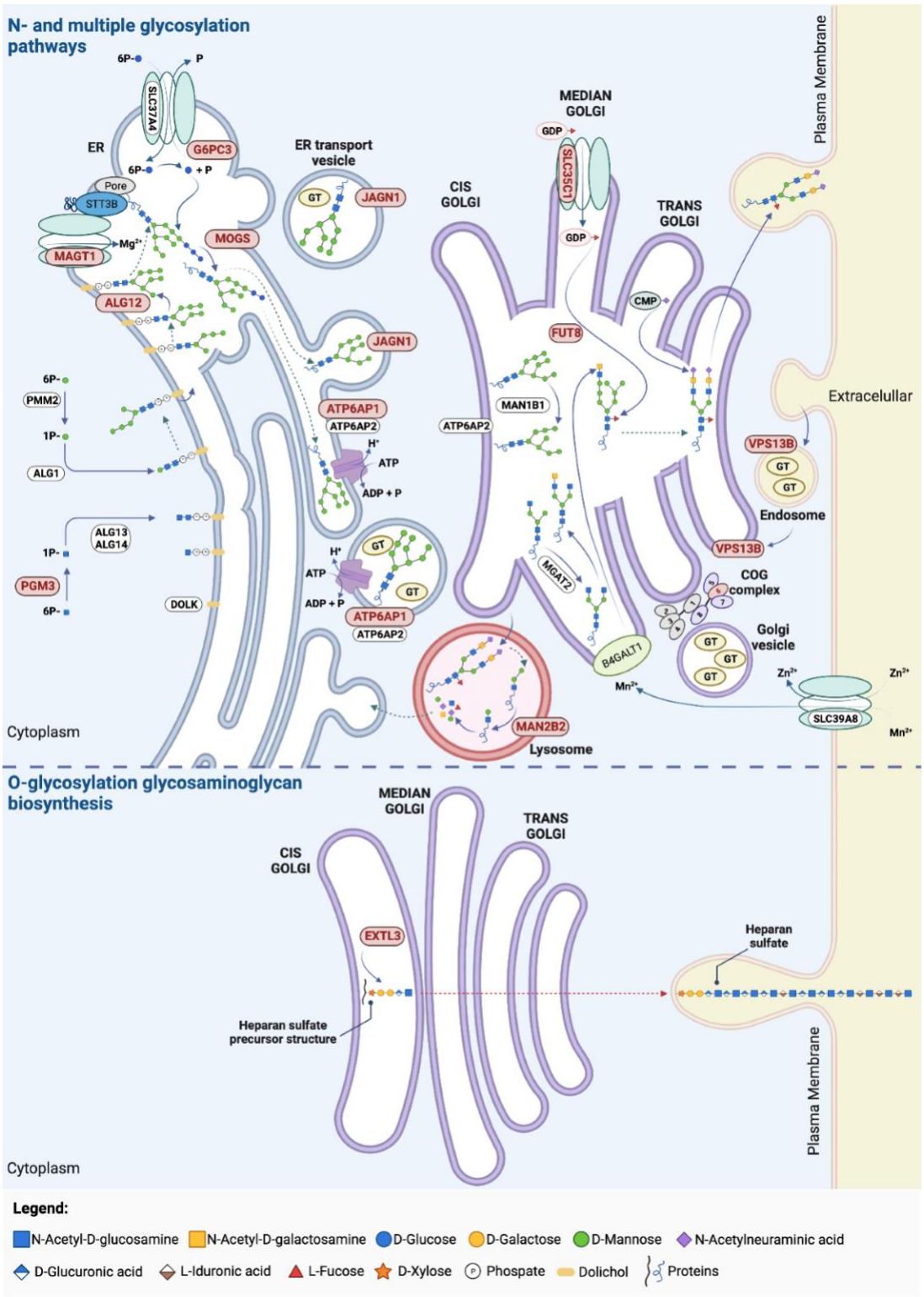

Figure 2.7. Glycosylation steps of the affected enzymes in CDG with major immunological involvement (in red). The illustration is divided in two parts. On the upper side, defects related to N- and multiple glycosylation pathways are presented. On the lower side, B) O-glycosylation glycosaminoglycan biosynthesis is depicted. Several steps and enzymes have been omitted to simplicity the representation.

Table 2.3. Immune manifestations of the 12 CDG with predominant immunological involvement.

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
N-linked defects							
ALG12-CDG (#607143) n= 12/19	Recurrent and/or severe infections (12/19): RTI (10/19), including RTI (1/19), pneumonia (5/19), otitis (2/19), and ear/nose infections (1/19) Sepsis (1/19)	Most infections identified as being bacterial Mild viral RTI (1/19) Haemolytic *E. coli* (1/19)	Aseptic meningitis (1/19) Necrotizing enterocolitis (1/19)	↓ B cells (1/19)	Hypogammaglobulinemia (1/19) ↓ IgG (9/19) with abnormal IgG N-glycans (1/19) ↓ IgM (3/19) ↓ IgA (1/19) ↑ IgG (1/19)	Absent response to diphtheria, tetanus, and H. influenzae vaccine (1/19) Mother's milk intolerance (1/19)	[124], [127]–[133]
FUT8-CDG (#618005) n= 8/9	Recurrent and/or severe infections (7/9): RTI (7/9), including pneumonia (2/9), bronchopneumonia (1/9) and pulmonary infections (1/9) accompanied by respiratory failure (apnoea/ hypopnoea)	NA	Reactive airway disease (1/9)	Neutropenia (1/9)	↓ IgG with severe hypofucosylation (2/9)	NA	[134]–[136]
JAGN1-CDG (#616022) n=25/26	Recurrent and/or severe infections (25/26): Abscesses (including skin, genital and oral) (15/26) Upper RTI (8/26), pneumonia (13/26), otitis (8/26), ear, nose, and throat infections (4/26), aphtosis, mouth ulcers, periodontitis (5/26) Cellulitis (5/26) Cutaneous infections (2/26)	*E. coli (2/26)* *Aspergillus* genus (1/26) *P. aeruginosa* (1/26) *Candida* genus (2/26) *K. pneumonia* (1/26) *Enterococcus* MDR (1/26) *M. morganii* (1/26) *S. aureus* (1/26) Gram negative bacillus (1/26) H. influenzae (1/26)	Gingivitis (6/26) with anti-thyroglobulin gingivitis (1/26) Pneumonitis (2/26) Bronchitis (2/26) Balanitis (1/26) Periodontopathic (1/26) Pancolitis (1/26) Lymphadenopathy (1/26) ↑ CRP (1/26)	Neutropenia (25/26) with ↓ RTEs (2/26) Monocytosis (2/26) Lymphopenia and ↓ class switched B cells and ↓ CD4 (2/26) Leukopenia (1/26)	Hypogammaglobulinemia (2/26) ↓ IgA (2/26) ↓ IgG (2/26) ↓ IgM (2/26)	BM arrest (24/26) Dust mite allergy (1/26) LTT test with decreased BCG, PHA, and candida response (1/26) Non protective anti-tetanus IgG and anti-diphtheria titres (1/26)	[137]–[140]

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
	Sepsis (3/26) Omphalitis (4/26) Onycholysis (1/26) Lymphadenitis (1/26) Dacryocystitis (1/26) UTI (1/26) GI tract infections (1/26)						
MAGT1-CDG (#300853, #301031) n= 35/51	Recurrent and/or severe infections (36/51): RTI (36/51), including sinusitis, otitis, pharyngitis, epiglottitis and pneumonia Skin infections (17/51), namely skin warts or condylomata acuminata Recurrent mouth sores (11/51) Meningoencephalitis (1/51) Sepsis (2/51)	EBV (43/51), with persistently elevated EBV-viremia (21/51) *M. contagiosum* (11/51) *Streptococcus* genus *(2/51)* *P. jirovecii (1/51)* HPV (7/51) HVS (6/51) VZV (5/51) CMV (2/51) JCV (1/51) Metapneumovirus (1/51) Parainfluenza Type 1 (1/51)	Severe autoimmune cytopenia (8/51) Guillain-Barre syndrome (5/51) Immune thrombocytopenia (4/51) Autoimmune hepatitis (3/51) Autoimmune haemolytic anaemia (3/51) Eosinophilic oesophagitis (1/51) Chronic bronchitis (1/51) Episcleritis (1/51) Alopecia partialis (1/51) Erythema multiforme (1/51) Lymphadenopathy (6/51) and lymphadenitis (2/51), with reactive hyperplasia of the lymphoid tissue (1/51)	Lymphopenia (2/51) T cell lymphopenia (4/51), ↓ $CD4^+$ (25/51) and ↓ $CD8^+$ (2/51) ↓ B cells (1/51) Lymphocytosis (3/51) ↑ T Cells (1/51) ↑ B cells (32/51) ↓ CD4/CD8 ratio (30/51) ↑ % of transitional B cells (CD24hiCD38hi) (1/51) ↓ switched memory B cells (1/51) ↓ class-switched plasmablasts (1/51) CD5+/CD19+ B cell predominance (1/51) ↓ $CD27^-IgD^-$ b cell switched (1/51) ↓ $CD27^+IgD^+$ non-switched B cell (1/51) ↑ B cell $CD27^-$ naive (1/51) ↑ αβDNTs (20/51)	Hypogammaglobulinemia (3/51) ↓ IgA (28/51) ↓ IgG (26/51) ↓ IgM (1/51) ↑ IgG (1/51) ↑ IgE (1/51) ↑ IgM (1/51)	Absent to ineffective response to the pneumococcal polysaccharide (8/51), diphtheria (2/51) and tetanus (2/51) vaccines Hodgkin's lymphoma (1/51) B cell lymphoma (3/51) Refractory non-Hodgkin lymphoma (1/51) Selective anti-polysaccharide antibody deficiency (1/51) Submandibulary and axillary lymphadenopathy with diffuse large B cell lymphoma upon excision (1/51)	[141]–[150]

CDG (#MIM) n=total n° patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
			Ulcerative colitis (1/51) Suppurative osteomyelitis (1/51) Bronchiolitis (1/51) Kawasaki disease (1/51)	↓ NKG2D surface expression on NK and $CD8^+$ cells (28/51) ↑ NK (1/51) ↓ NK cells (3/51) Transient neutropenia (14/51) and neutropenia (3/51) EBV-encoded RNA positive T cell muscle infiltration (1/51) ↓ expression of CD127 within $CD4^+$ cells (1/51)			
MAN2B2-CDG (NA) n= 1/2	Recurrent infections (1/2)	NA	Recurrent vasculitis (1/2) Recurrent arthritis (1/2) Positive rheumatoid factor (1/2)	T and B lymphopenia (1/2) ↓ naïve $CD8^+$ and $CD4^+$ T cells (1/2) ↑ terminally differentiated $CD8^+CD45RA^+CCR7^-$ cells (1/2) Skewed repertoire of cytotoxic T cells (1/2) ↓ T cell proliferation (1/2) Undetectable TRECs (1/2) ↑ circulating plasmablasts (1/2) ↑ dysreactive B cells (1/2)	↓ IgM (1/2) ↓ IgA (1/2) ↑ IgE (1/2)		[151], [152]

CDG (#MIM) n=total n° patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
MOGS-CDG (#606056) n= 29/29	Recurrent and/or severe infections (23/29): RTI (11/29) including pneumonia (9/29) Sepsis (6/29) Meningitis (2/29) UTI (2/29) Osteomyelitis (2/29) Tracheostomy/port infection (2/29) Nasolacrimal abscess (1/29) Cellulitis (1/29) Bacterial enteritis (1/29)	*E. coli* (2/29) *Mycoplasma* genus (2/29) *Serratia* genus (2/29) and *S. marcescens* (1/29) *P. Jirovecii* (2/29) *A. baumanii* (1/29) *E. cloacae* (1/29) *S. pneumoniae* (1/29) *S. aureus* (1/29) *Pseudomonas* genus (1/29) *Klebsiella* genus (1/29) and *K. pneumoniae* (1/29) *Corynebacterium* genus (1/29) *C. difficile (1/29)* *Achromobacter* genus (1/29) Methicillin-susceptible *S. aureus* (1/29) Viral origin (10/29), including rhinovirus/enterovirus (3/29,) RSV (2/29), CMV (1/29) and HPV 3 and 4 (1/29)	Enteritis (1/29) Poor wound healing (1/29) ↑ IL-6 (1/29) ↑ CRP (1/29)	B and T lymphocytosis with ↓ lymphocytic proliferation (2/29) Lymphocytosis (2/29) ↑ B cells (2/29) Leukocytosis (2/29) Neutropenia (2/29) ↓ T cells (1/29) ↑ % of neutrophils (1/29)	Hypogammaglobulinemia (22/29) ↓ IgA (19/29) ↓ IgM (7/29) ↓ IgG (13/29) with ↓ half-life with ↓ FγRIIa affinity (2/29) EBV-IgG (1/29)	Ineffective response to the measles, mumps, rubella, and varicella vaccine (2/29) ↓ anti-meningococcal IgG and anti-pneumococcal IgG (1/29) ↓ haemophilus influenzae B titre (1/29) Food allergy (2/29)	[124], [153]–[157]
O-linked defects							
EXTL3-CDG (#617425) n=13/16	Recurrent and/or severe infections (6/16):	*Klebsiella* genus (1/16) *S. aureus* (2/16) *Candida* genus (1/16)	Exfoliating dermatitis (Omen type) (5/16)	$T^-B^+NK^+$ (9/16) with ↓ T cell proliferation (3/16)	Hypogammaglobulinemia (5/16) ↓ IgG (2/16)	Absent response to the pertussis vaccine (1/16)	[158], [159]

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
	Upper RTI (2/16), pneumonia and other pulmonary infections (2/16) Sepsis (2/16) Omphalitis (1/16) Recurrent dental caries propensity (1/16) Oral infections (1/16)	Upper RTI infection identified as being of viral origin (2/16) CMV (1/16)	Chronic blepharitis (1/16) Pneumonitis (1/16)	Idiopathic CD4 lymphopenia with no naive T cells (2/16) ↓ T cells (1/16) Mild lymphopenia (1/16) ↓ or undetectable TRECs (4/16) Eosinophilia (7/16)	↓ IgM (1/16) ↓ IgA (1/16) ↑ IgE (3/16) ↑ IgG (1/16) ↑ IgM (1/16)		
Multiple glycosylation pathways defects							
ATP6AP1-CDG (#300972) n=31/36	Recurrent and/or severe infections (24/36): RTI, including pneumonia, bronchiolitis, and otitis (11/36) Sepsis (2/36) Plantar abscesses (5/36) GI infections (5/36) UTI (1/36) Ascites (2/36) Bacteriemia (1/36) Peritonitis (1/36) Cholangitis (1/36)	*E. coli* (2/36) *S. pneumoniae* (1/36) RSV (1/36) SARS-CoV-2 (1/36) Several viral infections reported in one patient (1/36)	Lymphadenopathy (1/36) Diffuse active colitis (1/36)[a] Gastritis (1/36)[a] Dyspeptic syndrome associated with fever (1/36)[a] Idiopathic hepatitis (1/36)[a] Febrile seizure (1/36) ↑ CRP (2/36)[a]	Lymphopenia (1/36) ↓ $CD4^+$ and $CD8^+$ T cells (1/36) ↓ $IgD1/CD27^+$ intermediate and switch memory cells indicative of defective B cell differentiation (2/36) Leukopenia (6/36) Neutropenia (1/36) Pancytopenia (2/36) Lipid-laden macrophages in liver biopsy (2/36) Vacuolated lymphocytes (1/36)	Hypogammaglobulinemia (22/36) ↓ IgG (12/36) ↓ IgM (7/36) ↓ IgA (5/36)	Ineffective response to the measles, mumps, rubella, and varicella vaccine (1/36) Some patients responded very poorly to vaccination[b], related to specific anti-polysaccharide antibody deficiency (1/36) Liver fibrosis with more infiltratng lymphocytes and plasma cells in the confluent area (1/36)	[160]–[163]
G6PC3-CDG (#612541) n= 153/153	Recurrent and/or severe infections (137/153): RTI (27/153), including otitis (43/153), pneumonia (35/153), pharyngitis (16/153), aphthous	*E. coli* (7/153) *Aspergillus* genus (3/153) *Candida* genus (2/153)	IBD, including Crohn's disease (25/153) Colitis (5/153) Gastroenteritis (24/153) Oral ulcers (20/153) Gingivitis (9/153)	Neutropenia (150/153) with ↑ neutrophil ER stress (5/153), hypogranular neutrophils with rare döhle bodies (1/153)	↑ IgG (22/153), namely during flares ↑ IgM (2/153) ↑ IgA (1/153) Hypogammaglobulinemia (2/131)	BM maturation (55/153) with arrest at the stage of promyelocyte/ myelocyte (20/153) BM hypercellularity (8/153) Asthma (5/153)	[38], [164]–[180]

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
	stomatitis (17/153), periodontal disease (6/153), dental caries (2/153), oral infections (2/153), bronchiolitis (1/153) and tonsillitis (2/153) Sepsis (28/153) UTI (15/153) including bladder infections (1/153) and pyelonephritis (1/153) Abscesses (22/153) Cellulitis (11/153) GI infections (2/153) Perianal infections (1/153) Osteomyelitis (1/153) Paronychial nail infections (1/153), Conjunctival infections (1/153), Omphalitis (4/153) Furunculosis (1/153) Mastoiditis (1/153) Sinusitis (2/153)	*Pseudomonas* genus (1/153) and *P. aeruginosa* (3/153) *S. aureus* (4/153) *S. viridans* (1/153) *K. pneumoneae* (1/153) *H. pylori* (1/153) *C. difficile* (1/153) VZV (2/153) RSV (1/153) Parainfluenza (1/153) HSV (1/153)	Recurrent proctitis (1/153) Panniculitis (3/153) Gastritis (4/153) Typhlitis (1/153) Celiac disease (1/31) Esophagitis (1/153) Blepharitis (1/153) Endocarditis (1/153) Rhinitis (1/153) Bronchitis (17/153) Parotitis (2/153) Recurrent fever (8/153) Pneumonitis (3/153) Genital aphthous ulcerations (4/153) Eczematous rash (1/153) Transient presence of anti-neutrophil and anti-HLA antibodies (1/153) Erythema nodosum (1/153) Familial Mediterranean Fever episodes (2/153) Necrotizing enterocolitis (1/153) Juvenile rheumatoid arthritis (2/153) ↑ CRP (2/153)	and impaired neutrophil motility and activity (1/153) Monocytosis (6/153) Leukopenia (17/153) Lymphopenia (25/153) with ↓ Pan-T cell (2/153) ↓ $CD3^+$ cells (3/153) ↓ $CD4^+$ cells (8/153) ↓ naive T cells (7/153), ↓ B cells (8/153) ↓ RTEs (3/153) ↓ NK cells (5/153) ↑ NK cells (5/153) ↑$CD16^+$ and $CD56^+$ (1/153) Pancytopenia (2/153) ↓ proliferation after mitogen stimulation (3/153) ↑ IL-1β and IL-6 (2/153) following LPS stimulation ↑ TNFα (on anti-TNFα therapy) (1/153)	↓ IgE (3/153) ↓ IgA (1/153) ↓ IgG (2/153)	Ineffective response to the tetanus (1/153) and H. influenza vaccines (1/153) Cow milk allergy (1/153) Medullar aplasia (1/153) Papillary thyroid carcinoma (1/153)	

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
PGM3-CDG (#615816) n=61/62	Recurrent and/severe infections (61/62): RTI (41/62), including pneumonia (22/62), chronic sinopulmonary disease (7/62), otitis (21/62) with tympanic membrane perforation (1/62) Skin infections (42/62), namely abscesses (16/62), impetigo, infected skin ulcers (3/62), eczema (10/62) and oral (7/62) infections Viral infections (14/62), including severe varicella (2/62), Soft tissue infections (7/62), including abscesses (9/62) Sepsis (7/62) Perichondritis (1/62) Osteomyelitis (1/62) Intrauterine bacterial infection (1/62) UTI (1/62) GI tract infections (2/62)	*Staphylococcus* genus (14/62), including *S. aureus* (6/62) and *S. epidermidis* (2/62), *Candida* genus (19/62) *Pseudomonas* genus (5/62) *K. pneumoniae* (1/62) *S. pneumoniae* (1/62) *E. cloacae* (1/62) *S. dysgalactiae equisimilis* (1/62) *Salmonella* genus (1/62) VZV (6/62) RSV (4/62) EBV (3/62) HPV (4/62) HVS (1/62) Influenza (1/62)	Eczema/dermatitis (40/62) often with pyodermatitis (15/62) Rhinitis (10/62) Bronchitis (4/62) Esophagitis (1/62) TSH receptor and TPO autoantibodies (1/62) Folliculitis (1/62) Autoimmune haemolytic anaemia (1/62) Pericarditis (1/62) Dorsal skin inflammation (1/62) Conjunctivitis (1/62) Graft versus host disease (1/62) Bullous pemphigoid (1/62) Blistering skin lesions (2/62) Atopic dermatitis (2/62) ↑ CRP (1/62)	Leukopenia (14/62) Leukocytosis (3/62) Neutropenia (15/62) Neutrophilia (2/62) Eosinophilia (28/62) Lymphopenia (38/62), including: ↓ T, B and NK cells (3/62) $T^-B^-NK^+$ (3/62) ↓ $CD3^+$ (13/62), $CD4^+$ (24/62) and $CD8^+$ (11/62) cells Reverted CD4/CD8 ratio (20/62) Th2 predominance and ↑ activated effector cells (7/62) ↓ naïve T cells and TRECs (5/62) Abnormal TRECs (1/62) ↓ % of $CD4^+CD45RA^+$ T cells (1/62) ↑ % of $CD4^+CD45RO^+$ memory T cells (1/62) ↓ T cell proliferation (16/62) ↓ $CD45^+$ total lymphocyte proliferation (1/62) ↓ NK cells (3/62) ↑ NK (8/62)	Hypogammaglobulinemia (1/62) ↓ IgE (1/62) ↓ IgM (5/62) ↓ IgA (3/62) ↓ IgG (2/62) ↑ IgE (46/62) ↑ IgM (5/62) ↑ IgG (7/62) ↑ IgA (9/62)	Hypocellular BM (4/62) Defective T cell development with ↑ thymic DNTs (1/62) Asthma (7/62) Food allergies (20/62), including cow milk (4/62) Drug allergies (3/62) Inadequate responses to the 23-valent pneumococcal polysaccharide vaccine (1/62) Respiratory related allergy (7/62) Other allergies (6/62)	[38], [181]–[189]

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
				↓ B cells (19/62) ↓ $CD20^{+}CD27^{+}$ cells (7/62) ↑ transitional B cells (5/62)			
SLC35C1-CDG (#266265) n= 20/20	Recurrent and/or severe infections (17/20) although their severity and recurrence was widely variable: RTI and pulmonary infections (11/20), namely pneumonia (3/20), bronchiolitis (1/20), otitis media (7/20), sinus infection (1/20), tonsillitis (1/20), mild to severe chronic periodontitis (8/20), recurrent dental cavities (2/20) Severe and/or localized cellulitis (5/20) Conjunctivitis (1/20) UTI (1/20) Omphalitis (1/20) Abscesses (1/20) Impetigo (1/20)	Coronavirus (1/20) HSV (2/20)[c] Also, in (1/20) infections were identified as being bacterial	Gastroenteritis (2/20) Inflammatory skin disease (1/20) Celiac disease (1/20) Aseptic meningitis (1/20) Recurrent unexplained fever episodes (4/20) ↑ CRP (1/20)	Leukocytosis (11/20) Neutrophilia (7/20) with deficient granulation (1/20) ↑ B and T cells (1/20) ↓ neutrophilic mobility (6/20)	IgG defective fucosylation (1/20) ↑ IgA and IgM (1/20)	Absent sLe^{x} and H antigen on erythrocytes (Bombay blood group) (8/20) Egg allergy (1/20)	[124], [190], [191]
VPS13B-CDG (#216550) n=169/284[d]	Recurrent and/or severe infections (63/284): Recurrent oral infections (46/284), including aphthous ulcers and gingivitis/periodontitis	*NA*	Juvenile rheumatoid arthritis (1/284) IBD (1/284) Lichen planus (1/284)	Neutropenia (150/284) Leukopenia (11/284) Monocytopenia (4/284) Monocytosis (1/284)	NA	No BM alterations were found in any of the studied patients Positive for anti-cardiolipin IgG and anti-beta-2-glycoprotein 1 IgG (1/284)	[38], [192]–[211]

CDG (#MIM) n=total nº patients	Clinical parameters			Biochemical parameters		Other (e.g., vaccination response/allergies)	References
	Recurrent/Severe infections	Pathogens	Autoimmune/ Inflammatory signs	WBC counts and functional parameters	Igs		
	Recurrent RTI (25/284), including ear, nose and throat (13/284), pneumonia (7/284), bronchitis (1/284), meningitis (1/284), ethmoiditis (1/284), and rhinopharyngeal infections (1/284) Skin infections (8/284), including skin warts (4/284) and cellulitis (1/284) Stomach infection (1/284) UTI (3/284) including pyelonephritis (1/284)			Pancytopenia (1/284)			

Legend: ↓ - Low or decreased; ↑ - high or increased; BM - bone marrow; CMV – Cytomegalovirus; CRP – C-reactive protein; DNTs - Double negative T cells; EBV – Epstein-Barr virus; ER – Endoplasmic reticulum; GI - Gastrointestinal; HLA – Human Leukocyte Antigen; HPV – Human papilloma virus; HVS – Herpes virus simplex; IBD – Inflammatory bowel disease; Ig – Immunoglobulin; IL - Interleukin; JCV - John Cunningham virus; LPS – Lipopolysaccharide; LTT - Lymphocyte transformation test; MDR – Multi-drug resistant ; NA – Not available ; NK – Natural killer; NKG2D - Natural killer group 2, member D; PHA - Phytohemagglutinin; RTI – Respiratory tract infections; RSV - Respiratory syncytial virus; RTEs - recent thymic emigrant; sLe^x - Sialyl Lewys X; TPO - Thyroid peroxidase antibodies; TNF - Tumor necrosis factor; TRECs – T cell receptor excision circles; TSH – Thyroid stimulating hormone; UTI – Urinary tract infections; VZV – Varicella Zoster virus ; WBC – Whole blood counts

[a] Complication found in the patient reported by [163] during immunosuppressive treatment following liver transplantation.

[b] [212] does not disclose the exact number of patients who did not respond to vaccination

[c] In SLC35C1-CDG patients, most often no pathogens could be identified during infection/fever episodes.

[d] The most often intermittent nature of the neutropenia found in VPS13B-CDG patients in addition to the fact that often blood cell counts are not always reported or available but also because of the generalized lack of severe and/or recurrent infections in these patients makes it highly likely that immunological manifestations, especially neutropenia, are underdiagnosed in VPS13B-CDG.

2.3.1.1. Immunodeficiencies affecting cellular and humoral immunity (I)

Recently, the newly reported MAN2B2-CDG has been included as an inborn error of immunity with the first patient presenting immunodeficiency, dysmorphic facial features, coagulopathy, and a severe developmental delay [151].

MAN2B2 is a member of the mannosidase gene family involved in the lysosomal degradation of glycoproteins, specific for the cleavage of the α1–6-Man residue of N-linked glycans. MAN2B2 deficiency seems to dysregulate the deglycosylation and monosaccharide recycling process and lead to abnormal mannosylation of glycans [151]. The resulting immunological clinical manifestations in the first patient included recurrent episodes of vasculitis, arthritis, and infections. Biochemically, T lymphopenia was noted with decreased counts of naïve cytotoxic and helper T cells, as well as a significant high proportion of terminally differentiated $CD8^{+}CD45RA^{+}CCR7^{-}$ cells, a skewed repertoire of cytotoxic T cells, undetectable T cells receptor excision circles (TRECs) and decreased T cell proliferation (Table 2.3). Additionally, B cell lymphopenia with high circulating plasmablasts and dysreactive B cells were detected with low IgM and IgA levels and high IgE. Autoimmunity was also observed with positivity for rheumatoid factor [151]. Nevertheless, besides some similar features, a newly reported Chinese presented normal immune parameters with additional presentations including cleft palate, bulging of occipital bone and hypospadias [152]. Therefore, it remains to be understood if this is indeed an inborn error of immunity and a CDG with major immunological involvement or a clinically heterogenous disorder with some or infrequent immunological occurrences [126].

2.3.1.2. Combined immunodeficiencies with associated or syndromic features (II)

EXTL3- and PGM3-CDG are part of the combined immunodeficiencies with syndromic features presenting with neurodevelopmental and skeletal defects. In EXTL3-CDG, the transfer of GlcNAc to glycosaminoglycan chains and the heparan sulphate biosynthesis is compromised. The immunophenotype is quite variable ranging from isolated infection occurrences to severe immunodeficiency, but some patients do not present any immune issues [213]. The reason for such clinical variability is still to be determined but it is thought that the immunodeficiency derives from early T cell development defects suggested by thymus assessment of a zebrafish model and inexistence of EXTL3 expression in circulating T cells, in contrast with haemato- and/or lymphopoietic cells [214]. In PGM3-CDG, there is an impairment on the synthesis of UDP-GlcNAc due to the deficient interconversion of GlcNAc-6-P into GlcNAc-1-P. This defect seems to affect all different types of immune cells in terms of counts and/or function and translate in repeated dermal and pulmonary bacterial or fungal infections (Table 2.3). Specifically, in this CDG, the N- and O-glycosylation of T cells is impaired and interferes with their proliferative capacity and cytokine production activity due to the dysregulation of several signalling pathways, namely NFAT,

NF-κB and STAT3-mediated signalling [215]–[218]. In turn, the abnormal levels of several cytokines (e.g., IL-6, IL-17, IL-27 and granulocyte colony-stimulating factor (G-CSF)) lead to defects in other cytokine-mediated immune responses (e.g., production of memory T and B cells), and to elevated IgE – a hallmark of PGM3-CDG – manifesting as allergic and atopic manifestations [218]–[221]. A dysregulation of the metabolic profile of PGM3-deficient cells was also postulated as a factor behind the immune issues observed [220].

2.3.1.3. Predominantly antibody deficiencies (III)

ALG12-, ATP6AP1-, and MOGS-CDG fall under the predominantly antibody deficiencies category since all these diseases present hypogammaglobulinemia, mainly low IgG counts (Table 2.3). Despite this common immune hallmark, they have distinct clinical presentations: while ALG12- and MOGS-CDG are multi-system disorders with development delays, hypotonia, dysmorphism and other organ-related manifestations, ATP6AP1-CDG mainly lead to an immunodeficient phenotype with liver involvement (Supplementary File 2.1).

While ALG12 enzyme adds the eighth Man to the lipid-linked oligosaccharides under construction, MOGS is responsible for the trimming of the first Glc residue from the Glc3-Man9-GlcNAc2 precursor, and defects in these proteins both compromise N-glycan biosynthesis [222], [223]. In turn, ATP6AP1 codifies the first accessory subunit of the proton transporting vacuolar (V)-ATPase pump and defects in this protein cause not only defects in N-glycosylation, but also in mucin type O-glycosylation [224], [225]. These defects are probably in the genesis of the antibody deficiency that occurs in these disorders. In fact, antibody glycosylation is known to modulate its stability and function. Specifically, deficient IgG glycosylation impairs the binding to some Fc receptors which control IgG half-life [226]. Accordingly, abnormal IgG glycosylation and/or reduced Ig half-life has been reported in ALG12-CDG and MOGS-CDG patients [227], [228]. This mainly translates in a propensity to recurrent and severe bacterial infections as well as altered lymphocyte counts and dysfunction. ALG12-, ATP6AP1-, and MOGS-CDG patients also show some events of inflammatory episodes absent responses to vaccinates (Table 2.3). While in MOGS-CDG this abnormal vaccination response has been associated to resistance to glycosylation-dependent enveloped viruses, a recent report challenges this hypothesis by identified several infections by enveloped viruses in 2 patients [222], [229]. Resistance to some viral infections in ATP6AP1-CDG is also probable given the role of the V-ATPase pump in the replication of HIV and rhinovirus, however, if this is an effect of glycosylation alterations is still to be elucidated [230], [231]. Besides glycosylation defects, the immunological dysfunction in this CDG is also thought to be influenced by the dysregulation of intracellular acidification, a relevant process to phagocytosis.

2.3.1.4. Diseases of immune dysregulation (IV)

MAGT1 deficiency has been only recently recognized as a CDG after the unveiling of the role of the magnesium transporter 1 (MAGT1) protein in glycosylation [232]–[234]. Clinically, most patients present with an immune dysregulation disease named X-linked MAGT1 deficiency with increased susceptibility to EBV-infection and N-linked glycosylation (XMEN) defect. Specifically, patients present increased susceptibility to viral respiratory, oral, and skin infections and, in some cases, autoimmune conditions and alterations in vaccination responses (Table 2.3). Very recently, another variation of the XMEN phenotype was described in one patient particularly englobing muscle involvement characterized by myositis with immune infiltrates [235]. However, two patients have been previously reported with a different neurological phenotype without immune involvement [236].

The immune dysregulation in MAGT1-CDG with the XMEN phenotype seems to stem from glycosylation alterations of a set of immune-related molecules, particularly STT3B-dependent protein glycosylation. Specifically, the Mg^{2+} transport defect results in incomplete NKG2D glycosylation, reducing its stability and membranal expression and increasing its degradation, compromising the anti-EBV responses in NK cells and $CD8^{+}$ T cells [237]. Additionally, CD28 (a mediator of T cell co-stimulation) is also hypoglycosylated possibly impairing CD28-mediated cell signalling which affects several immune processes, such as leukocyte activation, movement, apoptosis regulation, cellular adhesion, and adaptive immune responses, ultimately leading to impaired T cell activation [237], [238]. Importantly, CD70 underexpression and hypoglycosylation was found. This molecule has been shown to predispose shown to predispose individuals to uncontrolled EBV infection [238]. Lastly, Ig hypoglycosylation has been observed, which can explain the hypogammaglobulinemia (mainly of IgA and IgG) which occurs in about half of the reported patients (Table 2.3) [232]. All in all, these observations not only support the MAGT1 deficiency association with CDG but also explain the increased EBV viremia and a higher risk of EBV-related B cell lymphomas and lymphoproliferative disorders.

2.3.1.5. Congenital defects of phagocytes (V)

SLC35C1-, G6PC3-, JAGN1-, and VPS13B-CDG are congenital defects related to phagocytic cells which, subsequently, manifest with frequent and severe infections caused by various pathogens, particularly affecting the skin, oral cavity, and respiratory tract (Table 2.3).

SLC35C1 is responsible for the Golgi apparatus transmembrane transport of GDP-fucose (Figure 1B). It is also called leukocyte adhesion deficiency type II since *SLC35C1* variants cause fucosylation deficiencies affecting the biosynthesis of selectin ligands and other glycoproteins important for leukocyte adhesion [239], [240]. This impairs leukocyte migration, homing, and binding to endothelial cells hampering the natural ability to fight infections [241]. The clinical phenotype has recently been expanded with milder presentations involving intellectual disability,

short stature and mild infections [242]–[244] and more severe patients presenting additional dysmorphia, immunodeficiency and the Bombay blood type [245], [246].

Genetic variants in *G6PC3*, *JAGN1*, and *VPS13B* cause different forms of severe congenital neutropenia (SCN). While G6PC3- and JAGN1-CDG show persistent neutropenia and bone marrow defects, VPS13B-CDG presents intermittent decreased neutropenia with normal bone marrow development and cellularity (Table 2.3). Besides, G6PC3-CDG and VPS13B-CDG patients present other syndromic features (Dursun and Cohen syndrome, respectively), while JAGN1-CDG presents solely as SCN [247], [248]. These and other phenotype differences are likely to arise from the distinct roles of these enzymes in the glycosylation process.

G6PC3 catalysis the hydrolysis of Glc-6-P into Glc in the ER – the last step of glycogenolysis. Not only does this lead to decreased levels of Glc and Glc-6-P in the cytoplasm, but also patient neutrophils show truncated and Gal-defective N- and O-glycans [249]. JAGN1 is involved in the vesicle-mediated transport in the glycosylation pathway. JAGN1 deficiency affects the processing and trimming of glycoproteins leading to increased Gal-α-1,3-Gal terminated triantennary glycans and significantly decreased biantennary glycans with end-standing sialic acid [250]. Lastly, VPS13B protein mediates lipids transfer between membranes at organelle contact sites. Defects in VPS13B are characterized by Golgi disorganization, underglycosylated early endosome antigen and lysosome-associated membrane glycoprotein 2, and N-glycan maturation defects [251]–[253].

Even though the direct causal glycosylation-related pathological effects are still elusive, several mechanisms have been proposed to, at least partially, explain the possibly multi-factorial neutropenic phenotypes. Firstly, increased neutrophil death associated with metabolic dysregulation, increased ER stress and apoptosis rates stress as well as decreased respiratory burst, calcium mobilization, and neutrophil survival factor - SERPINB1 – levels have been observed [249]–[255]. Secondly, neutrophil dysfunction related to abnormal glycosylation of proteins associated with neutrophil migration, adhesion and cytotoxicity defects were also reported [253], [254], [256]. Lastly, bone marrow maturation defects and myelokathexis are sometimes seen in these patients [249], [257], [258].

Besides neutrophils, other immune cells and antibodies can be altered (Table 2.3). In fact, Hagelkruys et al. detected abnormal B cell counts and function associated with altered ER homeostasis and aberrant Ig N-glycosylation in a Jagn1 deficiency mouse model and patient-derived cells [259]. Inflammatory and autoimmune manifestations also tend to occur, especially in G6PC3-CDG. In particular, IBD was found to be present in 16 % of G6PC3-CDG patients (Table 2.3). It was suggested that IBD prevalence in this CDG could derive from higher and consistent neutrophil activation leading to elevated expression of adhesion molecules, inflammatory cytokines, and reactive oxygen species [260]. Besides, IBD-affected G6PC3-CDG patients also showed upregulated platelet factor 4 serum levels, a Crohn disease's biomarker correlating with disease activity and treatment response [261].

2.3.1.6. Predominantly lung immune dysfunction

FUT8-CDG is caused by genetic variants in fucosylatransferase 8 which catalyses the transfer of the fucose residue from GPD-fucose to the first GlcNAc residue of the N-glycans - also known as core fucosylation. To date, 9 patients have been described and are affected by a severe multi-organ disease with neurological impairment, skeletal alterations, dysmorphic features, and GI manifestations [134]–[136]. Immunological involvement has been reported in 8 of them and seems to be mainly restricted to infection and inflammatory events of the lungs and respiratory tract (Table 2.3). In severe cases, these events may lead to respiratory failure [136]. Studies suggest that these localized lungs immune dysfunction observed in FUT8-CDG can be related to dysregulation of the TGF-β1 cytokine and the presence of emphysema-like lung abnormalities [262].

Cellularly and biochemically, the few reports only show the occurrence of neutropenia and IgG hypofucosylation in FUT8-CDG (Table 2.3). Nevertheless, FUT8-related depletion of core fucosylation has been linked to a direct impact on antibody function, profound alterations in B and T cells, lymphopenia, hypogammaglobulinemia as well as impaired recognition, assembly, and lipid raft association of pre- and IgG-B cell receptor antigen [263]–[267]. Even though these finding underline a key role of FUT8 in controlling lung immunity and inflammation, more detailed investigations are needed to: (1) possibly highlight a systemic immune response impact which might become apparent as more patients are diagnosed and (2) accurately categorize FUT8-CDG as an inborn error of immunity.

2.3.2. PMM2-CDG: a CDG with minor immunological involvement

Apart from the 12 CDG with major immunological involvement, several other CDG present less frequent (but not always less severe) immune issues. This is the case of phosphomannomutase 2 (PMM2)-CDG. PMM2-CDG is not only the most common N-glycosylation disorder, with an estimated incidence is 1:20 000 [268], but also the first ever described CDG in 1980 [269]. This CDG is caused by pathogenic autosomal recessive genetic variants in *PMM2* which affects PMM2 activity. Specifically, PMM2 catalyses the conversion of Man-6-P into Man-1-P, which is a precursor of guanosine diphosphate Man (GDP-Man) and dolichol-P-Man (Dol-Man). These two Man compounds are the donors of Man used in the ER for the dolichol-pyrophosphate oligosaccharide precursor assembly. Deficiency of GDP-Man and Dol-P-Man causes hypoglycosylation of numerous glycoproteins, including serum glycoproteins (lysosomal enzymes, and transport proteins) and membrane glycoproteins. This results in multi-organ involvement and in a broad spectrum of clinical presentations, ranging from severe neonatal to mild adulthood presentation. Almost all systems/organs can be involved in PMM2-CDG, with predominant neurological involvement, followed by ophthalmological, gastrointestinal, and

haematological involvement [268]. The pathophysiology and the variability of disease severity and course are not well understood.

Some PMM2-CDG patients present immunological dysfunction which range from infrequent and mild events to recurrent manifestations and fatal outcomes (Table 2.4). Recurrent infections are a common manifestation in early life and childhood (sometimes leading to sepsis) and become less frequent and less severe in adolescence and adulthood [270]–[272]. Regarding cellular and biochemical anomalies contributing to the clinical phenotype, hypogammaglobulinemia, T lymphopenia, leukocytosis and reduced neutrophil chemotaxis have been reported [271], [273]–[275]. Altered responses to vaccination have occasionally been described [271]. Infections were also reported to be triggers of stroke-like episodes [276]. Accordingly, PMM2-CDG families-reported data point out that infections are a cause for reduced quality of life due to frequent hospitalizations and triggers of other manifestations [272].

Currently, there are no targeted therapies for immune issues that afflict PMM2-CDG patients nor curative or effective treatments for PMM2-CDG in general. Prior to the identification of the underlying molecular cause of PMM2-CDG, Panneerselvam and Freeze had shown that the hypoglycosylation phenotype in PMM2-CDG patient fibroblasts could be resolved by the addition of Man to the culture medium [277]. This has been replicated *in vitro* [278]–[281] and *in vivo* models [282]. In PMM2-CDG patients, Man appeared to be well-absorbed, increasing the blood levels of this sugar without any associated renal and/or hepatic toxicity [283], [284]. Nonetheless, no clinical or biochemical improvement was observed in these patients [284]–[286]. However, Taday *et al* argued that long-term Man supplementation in PMM2-CDG might be of benefit [287]. Alternative therapeutic strategies under consideration or development are the use of pharmacological chaperones to stabilize PMM2 [288], [289], supplementation with liposomal-encapsulated Man-1-P [290] or under prodrug forms [291], [292], antisense therapy through morpholino oligonucleotides [293], blockage of alternative metabolic pathways [294], [295] or drug repurposing strategies [296]–[299]. Whether these approaches have a potential in the correction of the immunopathology of PMM2-CDG is still to be deciphered.

The lack of available therapies highlights the need to unravel the mechanisms behind PMM2-CDG (immuno)pathology, to allow therapy development and approval, answering a community-identified need.

Table 2.4. Clinical features and biochemical parameters of reported PMM2-CDG patients with immunological involvement.

Reference	Cases with immune involvement	Genetic variants (Va/Vb)	Age (at report)	Clinical Features		Biochemical Parameters	Observations
				Infections (pathogen, when identified)	Immune manifestations		
[300]	1	V129M/R141H	4 m		Recurrent episodes of fever related to hypogammaglobulinemia		Required Ig infusions
[301]	21	R141H/F119L	-	Some presented intercurrent infections with impairment of serum aminotransferases	Febrile seizures		Two patients did due to pneumonia (4.3 y and 6.0 y), one patient with septic shock (1.8 y), and another with unclassified infectious disease (9.9 y)
[302]	1	24delC in exon 1 leading to a frameshift /V231M	10 m	Three septic-like events in the first 8 weeks of life (inconclusive bacterial infection)	High fever	↓ CRP Leukocytosis	Serum transaminase concentrations highly increased during the septic like-events
[303]	6	-	-	Episodes of infection with fever and seizures			
[304]	1	N216I/N216I	16 m	Several upper airways infections Bronchopneumonia at 4 m			
[37]	2	-	-	-	Hypogammaglobulinemia		
[305]	1	-	3 m	Neonatal sepsis (*S. aureus*)	Persistent dermatitis		
[306]	1	E93A/R141H	6 Y	Upper tract urinary infection and frequent respiratory infections in the 2 first years of life		↑ white blood cell count with significant left shift	
[307]	1	F119L/F157S	Newborn		Macrophage activation with phagocytosis of erythropoietic elements (no signs of infection)		Died after 8 weeks of life
[308]	1	V231M/T237R	Newborn	Pneumonia at 1 week old (*Klebsiella*)			Died of circulatory and respiratory failure at 1 m

Reference	Cases with immune involvement	Genetic variants (Va/Vb)	Age (at report)	Clinical Features		Biochemical Parameters	Observations
				Infections (pathogen, when identified)	Immune manifestations		
[271]	2	-	-		↓ neutrophil chemotaxis Poor vaccine response		IVIG treatment
[309]	1	T237R/C241S	5 y		Intraepithelial lymphocyte infiltration without villous atrophy (type I lesion according to the Marsh classification) at 18 m		
[310]	1	F119L/F157S	-	Recurrent infections (bacteria)	↓ immunoglobulin levels		Died at 8 w because of severe failure to thrive and recurrent bacterial infections
[311]	2	24delC/P113L	6 y	-	Diffused cerebral vasculitis		
		R141H/V139M	8 y	-	Pericarditis		
[312]	1		6 y	Neonatal sepsis Gastroenteritis at 2 m Recurrent episodes of upper airways infection		Progressive lymphopenia	Cow milk intolerance
[313]	2	F119L/R141H	-	Infection with acute episode of ascites and pericardial effusion (adenovirus)			Died at the age of 6 y
		D188G/L35X	-	Minor infections with recurrent episodes of hypoalbuminemia, ascites, tachypnea, and dyspnea			Died 4 months later during an acute episode of overwhelming ascites production and pericardial effusion in multi-organ failure
[314]	1	M1V/V129M	-	Fatal lung infection at 3 m			
[315]	2	I132T/F207S	-	Few infections and bronchiolitis (viral)			
		R141H/V231M	11 m	-	Enterocolitis		
[316]	1	F157S/C241S	4 y 6 m	Chickenpox (viral)			

Reference	Cases with immune involvement	Genetic variants (Va/Vb)	Age (at report)	Clinical Features		Biochemical Parameters	Observations
				Infections (pathogen, when identified)	Immune manifestations		
[317]	2	R141H/F119L	12 y	Group A *Streptococcus*, *E. coli* (bacterial infections)			Secondary worsening of lymph vessel structure due to infections might play an important role in the clinical course
		R21G/R21G	29 y	Recurrent infections (bacterial; 2 m)			
[318]	1	G57R/R141H	3.5 m			Leukocytosis	
[319]	5	E33X/V44A	1 y	Severe viral infections (rotavirus at 3 m, complicated with post-natal cytomegalovirus infection)		↓ CD3+ ↑ CD16+/CD3+ ratio	
		R141H/V231M	1 y	Severe viral infections at 5.5 m (adenovirus) and at 1 y (rotavirus)		↓ CD3 ↑ CD16+ ↑ CD16+/CD3+ ratio	Died at 14 m due to multi-organ failure, after ICU admission due to infection
		Y64C/R141H	4 y	Severe viral infections: enteritis at 5 m (rotavirus) and 8 m (adenovirus) and bronchiolitis (respiratory syncytial virus) at 5 m Recurrent ear infections responsive to oral antibiotics		↓ CD3	
		P113L/ haploinsufficiency	4 y			↓ CD16+	
		P113L/ T118S+P184D	9 y			↓ CD3	
[320]	2	R141H/V231M	Deceased	Pneumonia (21 m)		T lymphopenia (↓ CD3, CD4 and CD8) Hypogammaglobulinemia (IgG and IgM)	Died at 21 m due to heart failure and pulmonary edema (fatal infection crisis)

Reference	Cases with immune involvement	Genetic variants (Va/Vb)	Age (at report)	Clinical Features		Biochemical Parameters	Observations
				Infections (pathogen, when identified)	Immune manifestations		
		R141H/G208A	Deceased	Episodes of bacterial sepsis (1 y)			Died at 2 y
[321]	1	I153*/I122T	8 m	Recurrent lower respiratory infections (1/month at 3 m) Recurrent lower respiratory infections (3/month at 3 y)		Persistent low CD4+ T cells; Persistent hypogammaglobuline mia (IgG)	IVIG treatment
[322]	3	L32R/IVS-1G>C	14 y 5 m	Enterovirus			Infection was a trigger of a SLE
		P113L/F207S	3 y 3 m	Influenza virus infection			Infection was a trigger of a SLE
		D65Y/R141H	5 y 8 m	Upper respiratory viral infection			Infection was a trigger of a SLE
[323]	1	L104V/L104V	6 y			Persistent leukocytosis (from 6 to 9 y)	
[324]	1	R141H/V231M	Deceased	Two febrile infection episodes at 10 m (during one of these episodes, *A. baumanii* was detected in the ascites)		Leukocytosis (6 w)	Developed respiratory insufficiency and died from cardiac failure following routine vaccination (11 m)
[325]	1	p.L82Vfs*2/ p.I132T			Febrile seizures		
[326]	1	g.18313A > T in exon 5 with 2 effects: homozygote for p.I132F/ p.I132F and p.G117Rfs*4 responsible for the skipping of exon 5	10 m	Presumably viral encephalitis (6 w)			Died at 10 m due to cardiac issues
[327]	1	F119L/F119L	21 y	Several infections (5y)	Febrile seizures (4y)		
[328]	1	p.C9AfsX27/ p.V129M	Deceased			Pancytopenia	Unknown cause of death

Reference	Cases with immune involvement	Genetic variants (Va/Vb)	Age (at report)	Clinical Features		Biochemical Parameters	Observations
				Infections (pathogen, when identified)	Immune manifestations		
[329]	1	D12H/L104V	23 y	Common cold (11y)			Common cold lead to hospital visit
[330]	2	P113L/P113L	13 y	Gastroenteritis (viral)	Refractory fever		
		V44A/R141H	24 y	Several unexplicit infections (see observation)			Infection with associated hyperthermia and epileptic seizures triggered recurrent episodes of somnolence and irritability of several hours' duration
[331]	2	G15R/R141H	Deceased	Recurrent infections Presumed meningitis (34m) Gastroenteritis (*Clostridium difficile* at 7m and Parvovirus at 46m) Sinusitis (46m)	Eczema Febrile illness	Pancytopenia	
		G42R/P113L	Deceased	Recurrent infections Pneumonia and sepsis (9 y)			
[332]	1	R141H/L32R	13 y	Recurrent upper and lower respiratory tract infections (3 y); pneumonia (4 y)			
[333]	1	G117C/I120T	2 m	Pneumonia			
[334]	3	Promoter variant in PMM2 (c.-167G > T) in trans with R141H	6 y		Eczema GI inflammation (oesophagus, stomach, duodenum, and throughout the colon) Colon moderate chronical active pancolitis, cryptitis, crypt abscess formation		

Reference	Cases with immune involvement	Genetic variants (Va/Vb)	Age (at report)	Clinical Features		Biochemical Parameters	Observations
				Infections (pathogen, when identified)	Immune manifestations		
		Promoter variant in PMM2 (c.-167G > T) in trans with R141H	10 y		Active gastric inflammation with neutrophils invading the glandular epithelium Chronic active pancolitis with cryptitis		
		Promoter variant in PMM2 (c.-167G > T) *in trans* with R141H	6 y		Eczema Eosinophilic oesophagitis Chronic active gastritis Active inflammation with cryptitis and crypt abscesses Type 1 hypersensitivity (anaphylaxis) to egg		
[335]	10	G15A/R141H	3 y	Infection simultaneously with deep venous thrombosis			
		P113L/V182D	7 y		Febrile illness		
		T237R/C241S	8 y		Febrile illness		
		R141H/N216I	3 y	Septic episode			
		P113L/T237R	7 y		High fever (associated with SLE)		
		R141H/F119L	29 y	Hospitalized with SARS-CoV-2 infection			
		R141H/V231M	Deceased		Febrile illness		
		R141H/F119L	1 y	Possible sepsis			
		R141H/P113L	9 y		Febrile illness		
		F119L/ *			Periods of fever associated with SLE		

Legend: CRP - C reactive protein; Ig - Immunoglobulin; IVIG - Intravenous immunoglobulin; m - Months; SLE - Stroke-like episodes; w - Weeks; y - years

2.3.3. Therapeutic options for the immunopathology of CDG

Very few CDG have approved and specific treatments. In general, the standards of care for CDG patients are limited to preventive and management interventions, which also applies for immunological complications.

Regarding infection treatment, intravenous (IV) or subcutaneous Ig administration has resulted in clinical improvement in many CDG patients [124], [212], [336]–[339]. Nevertheless, patients with hypogammaglobulinemia refractive to Ig therapy have been reported [340], [341]. Antibiotics– via IV or oral administration – have also been extensively used both as a preventive and curative measure. In general, a good response to antibiotic therapy (alone or in combination with Ig replacement therapy) has been observed [341]–[350].

Concerning autoimmune and inflammatory manifestations, anticholinesterase inhibitors and steroids have had fluctuating beneficial effects on CDG patients presenting with autoimmune myasthenia gravis [351], [352]. Anti-inflammatory and immunosuppressive agents have been employed to manage IBD in G6PC3-CDG [352]–[356]. Interestingly, in two GALNT3-CDG patients with generalized inflammation and foamy macrophages a treatment regimen with IL-1 antagonists resulted in decreased inflammatory markers, reduced tumour burden and improved general health in both subjects. Hence, inflammation markers (e.g., CRP levels and macrophage infiltration) should be monitored and targeted anti-inflammatories considered as an adjuvant treatment [357].

Therapeutic strategies to correct immune cell counts and/or function include leukocyte transfusion, steroids and G-CSF [345], [358]–[361]. Nevertheless, therapy resistance, lack of compliance, inconsistent effectiveness and/or side-effects (e.g., splenomegaly, giant cell tumours and acute myeloid leukaemia following high doses of G-CSF) have been reported in these patients [258], [340], [342], [354], [362].

In severe immunodeficiencies, hematopoietic stem cell transplantation (HSCT) has been performed [51], [342], [363], [364]. HSCT has resolved infections in a JAGN1-CDG patient, recovered normal T-cell development in an EXTL3-CDG patient and is now an approved therapy for PGM3-CDG in the USA, due to its outstanding clinical results in some patients [364], [365].

Nutrient supplementation with sugar precursors (e.g., Gal, fucose, Glc) has been attempted. In many cases, it was unsuccessful, as for instance the supplementation with UDP- GlcNAc in PGM3-CDG and fucose in FUT8-CDG [51], [134]. The exceptions are: (1) fucose administration in SLC35C1-CDG patients with well-defined mutations which significantly improved leucocyte migration defects, leukocytosis and resolved infections; and (2) Gal supplementation in combination with G-CSF in a SLC37A4-CDG patient that ceased infections and abscesses. These targeted approaches underpin the glycosylation defect as the underlying cause of the immune-

related alterations observed in patients and reinforce the necessity of targeted and more effective immune-therapies for CDG patients [239], [346].

2.3.4. Triple-negative breast cancer: clinical and immunological features

As of 2020, female breast cancer was the most diagnosed cancer and the lead cause of death globally [366]. It has been classified according to its expression of certain molecules, which has also been the basis for the development of targeted treatments [367]. Triple negative breast cancer (TNBC) is a term that encompasses all cancers that are negative for three receptors: estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2). These cancers make up around 15 to 20 % of all breast cancer types and are characterized by a high, still unclear, heterogeneity [367], [368]. Clinically, TNBC present moderate- to high-grade behaviour, translate mostly into invasive ductal carcinomas, have bad prognosis and high risk of early-recurrence [367]–[370].

Like other types, TNBC rely on the hallmarks of cancer to strive. Some of these hallmarks can be related to or resort to the immune system, particularly avoiding immune destruction, a tumour-promoting environment, and the ability to activate invasion and metastasis. In TNBC, there are several examples of mechanisms of immune evasion which overlap with the promotion of environments beneficial to the malignant cells. It has been shown to happen through the integrin αvβ6-mediated expression of SOX4, which lead to the activation of TGFβ- an important player of the tumoral immunosuppressive action [371]. Besides, tumours overexpressing the *MYC* gene hinder not only the expression of MHC-I, key to the presentation of tumour-associated antigens, and clearance by cytotoxic lymphocytes but also immune cell infiltration [372], [373]. Lastly, it was noted that the presence of the transmembrane mucin 1 leads to the expression of PD-L1 by increasing the recruitment of the transcription factors MYC and NF-κB (p65) to its promoter [374]. In turn, like in other tumours, PD-L1 inhibits T cell responses and consequently, tumour killing [375]. Concerning the activation of invasion and metastasis, it is well known that cancer cell appropriate immune-like mechanisms, specifically leukocyte transendothelial transmigration. This is the multi-step process that allows leukocytes to be recruited towards sites of inflammation, moving from the blood circulation to the body tissues by trespassing the blood vessels [376]. This process relies on a plethora of adhesion and signalling molecules that help the cells slowing down, attach to the vessels and suffer structural changes that allow them to perform diapedesis. An example of these molecules is the e-selectin, which typically bind to the tetrasacharide sialyl-lewis X (sLe^X) and A (sLe^A) and other related structures [377]. One of the ways cancer cells take advantage of this mechanism is by overexpressing these sialylated structures and misuse the ligation to e- and other selectins, gaining the capability to migrate to distal sites [378]. Even though there are no reports of the direct role of $sLe^{X/A}$ in TNBC, appropriation of transendothelial

migration is probable characteristic of TNBC since expression of $sLe^{X/A}$ is higher in metastatic breast cancers [379], [380].

Despite the recent approval of treatments for metastatic TNBC [381], [382], which efficacy is still far from desirable, due to its extreme heterogeneity, it remains the only group of cancers without targeted treatment.

2.3.5. People-centricity: an approach to drive basic and clinical research

For many decades, advocacy movements have been taking action to ensure people see their rights in place. The success of the efforts to improve healthcare is visible with patient-centred achievements continuously increasing [383]. The definition of patient- or people-centricity entails that citizens needs are prioritized by engaging them in a transparent and consistent way to obtain the best outcomes and experience for them and/or their families [384]. This new paradigm ensures that people's needs and preferences are considered, whether it is regarding their care, selected lines of basic research or design and engagement in clinical trials. Indeed, the benefits of a people-centric mindset in all aspects of basic and clinical research are also becoming increasingly recognized. In this field, the people are not only at the centre but rather are considered equal partners in the process of decision-making [385]. Emphasizing the importance of this new paradigm are initiatives from governmental and regulatory bodies which support, guide, and require public involvement and/or engagement in R&D [386], [387].

2.3.6. Public involvement across the R&D process

Public involvement (PI), previously referred to as public and patient involvement, should be differentiated from of sole participation (i.e., "taking part") and engagement ("interaction"). Instead, the concept of PI entails active partnerships between the public and researchers in the research process. In other words, PI is frequently outlined as doing research 'with' or 'by' people (…) rather than 'to', 'about' or 'for' them [388].

PI should be supported by a structured and comprehensive framework that contemplates the value of the people or patients' partnerships thought all steps of the research project or medicine development [386], [389]–[391]. In the context of basic research, there is not, however, a unique, widely accepted framework on which all stakeholders rely on as they differ on the context and purpose. Nevertheless, the National Institute for Health and Care Research - Research Design Service (NIHR-RDS) provides a clear and complete guidebook for researchers, describing the roles that patients and the public can have in eight steps of a research project: "identifying and prioritizing"; "design"; "development of the grant proposal"; "undertaking/managing"; "analysing and interpreting"; "dissemination"; "implementation"; and "monitoring and evaluation"

(Figure 2.8) [387]. Importantly, the significance of methods to evaluate this engagement has been highlighted to ensure that it is performed based on six key principles: clarity, reflexivity, methodological rigor, transparency, pragmatism, and reciprocity [392]. When referring to patient involvement in clinical research or drug R&D, the guidance introduced by the European Patients' Academy on Therapeutic Innovations is considered a best practice describing the types of involvement that people can have across all cycle of drug R&D as well as distinguishing between the degrees of disease expertise and capacity needed according to the task and phase (Figure 2.9) [393].

Within every PI framework, involvement is advised to start early and practiced consistently until project closure. Throughout the PI journey, the public can take on diverse roles depending on the desired direction, goals and expected excellence of the project. Patients and other members of the public can enrol in project advisory committees, assume research and/or reviewer functions, assume decision-making responsibilities and even be co-applicants [386], [387], [393]. The adoption of PI strategies brings, however, a need for literacy, capacity-building, and empowerment. Not only training exists to provide the required level of education and information to the lay public, patient, caregivers, and patient representatives, but also, initiatives are taking place to educate researchers and other professionals to engage patients and the public meaningfully [394]. Fruit of this increasing capacitation is the raise of patient groups-led and patient-initiate projects [395]. Similarly, the success of PI in different areas of basic and clinical research is increasingly visible [396]–[398]. Important outcomes benefiting both the public and researchers arise by the identification of new research paths, study and data quality optimization, maximization of recruitment and minimization of dropouts, between others, ultimately guaranteeing the validity and relevance of the research [399]–[402].

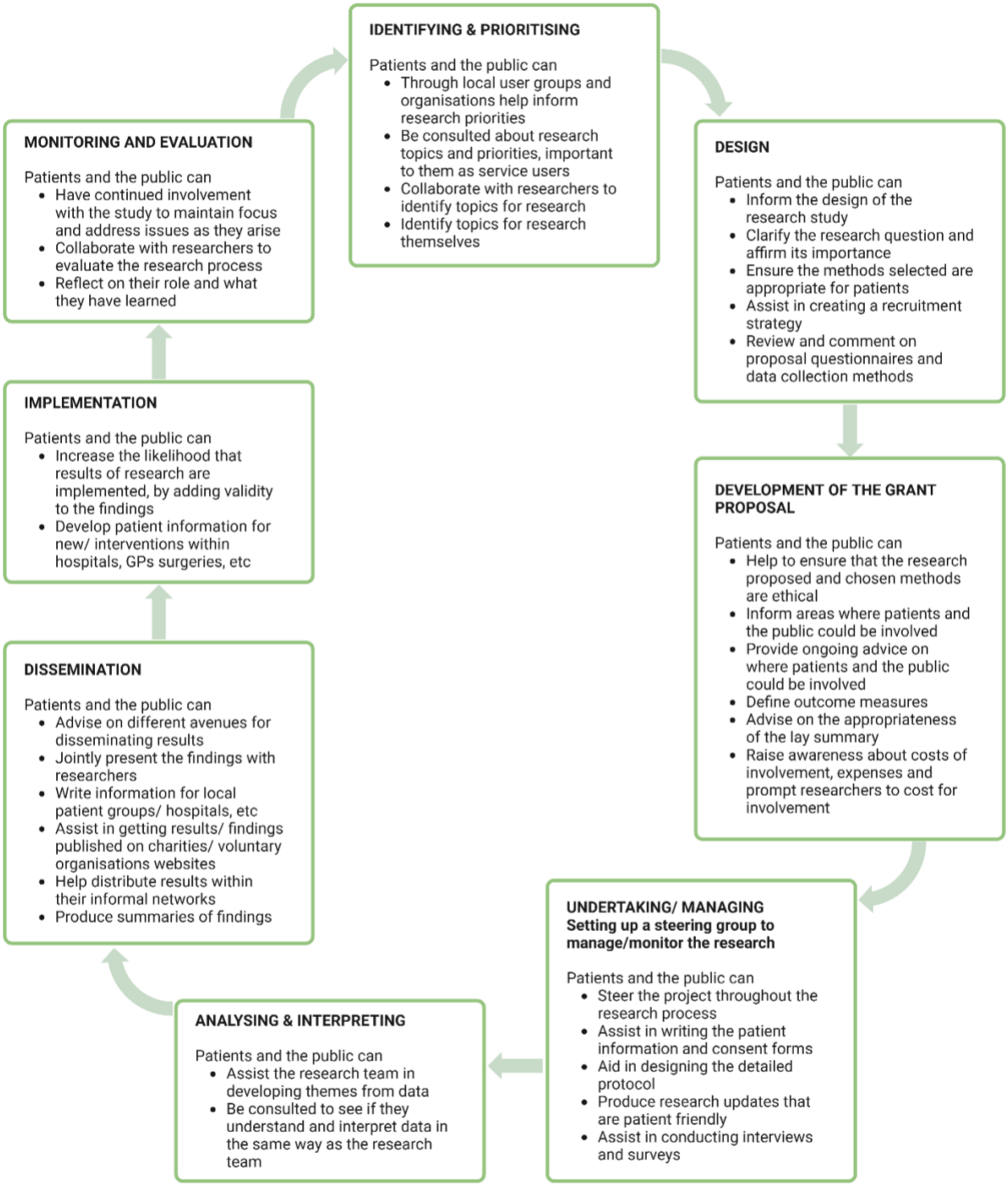

Figure 2.8. Guidance from the NIHR-RDS on how to incorporate public involvement into the research process. Adapted from [387].

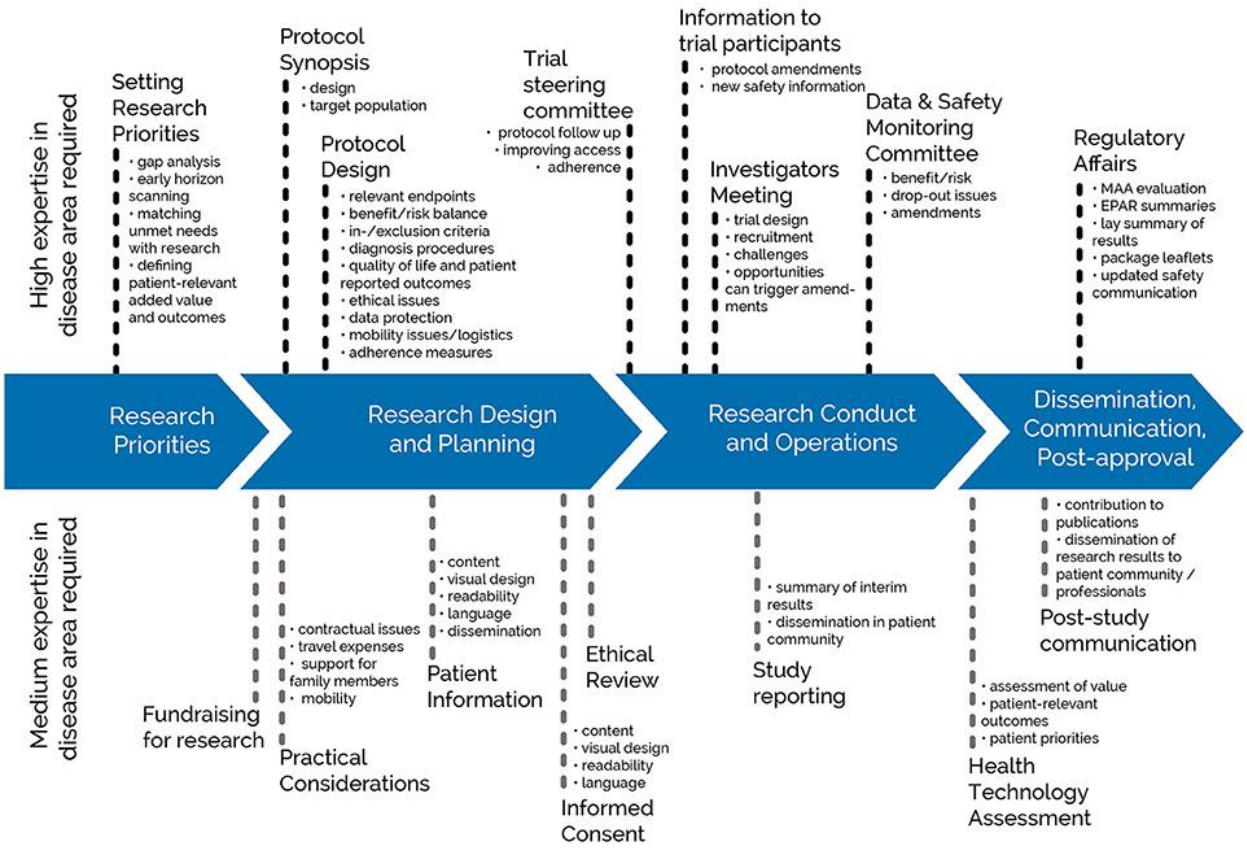

Figure 2.9. Guidance for public involvement in the drug R&D process, according to the European Patients' Academy on Therapeutic Innovations. Adapted from [393].

2.3.7. Capturing the patient experience with patient reported outcomes

While patient-centricity and PI are recent concepts, one of the ways in which these concepts have been advanced is through the development of clinical outcome assessment (COA) tools, ensuring that they are specific, adequate, targeted, and relevant for the targeted disease population. In fact, the correct development or selection of a COA can dictate the fate of an investigational drug approval success or failure. Particularly relevant in this setting are patient or observer reported outcome measures (PROMs or ObsROMs, respectively). PROMs are direct reports from patients regarding their health condition registered via validated questionnaires with robust psychometric properties [106]. ObsROMs are reports made by a proxy who is in direct contact with the patient when it is not possible to obtain self-reports [107].

PROMs have numerous applications in diverse settings, including research, policy decision-making or treatment effectiveness assessment [108]–[110]. Besides facilitating communication between patients and clinicians (which improves healthcare provision), they can also improve patient outcomes [111]. Importantly, the use of PROMs is highly supported by regulatory agencies,

such as the European Medicine Agency (EMA) and the Food and Drug Administration (FDA) [112], [113]. They are frequently used in clinical trials, mainly as surrogate endpoints [114], [115]. Among other parameters and variables, PROMs can be used to evaluate HrQoL [116]–[118]. While PROMs and ObsROMs are often used in common chronic conditions [119], [120], these tools have not often been applied or even developed in rare diseases, due to their specificities [121]–[123]. However, there are a few examples of the successful use of PROMs in rare diseases, particularly in academia, by PROM methodologists, and in collaboration with patient organizations [123], [124].

2.3.8. Health-related quality of life (HrQoL): a crucial health outcome

HrQoL is defined as a multidimensional concept referring to the subjective evaluation of the impact of the health status in domains related to physical, mental, emotional, and social functioning. It goes beyond direct measures of population health, life expectancy, and causes of death [403]. Rare and complex diseases are usually characterized by striking heterogeneity associated with a lack of evidence-based data and access to clinical experts. This makes it difficult for patients and caregivers to guide their decisions about disease management [404]. These factors have a tremendous impact on patients' health-related quality of life (HrQoL) [405].

In the case of PMM2-CDG, patients experience significant impacts in their quality of life due to the debilitating nature of the disease, as well as its psychological, financial and time burden [406], [407]. In fact, the neurological involvement that characterizes PMM2-CDG negatively correlated with activities of daily living which accesses functional ability [408]. Besides, the recent administration of the general Patient-Reported Outcomes Measurement Information System (PROMIS) to caregivers of PMM2-CDG patients identified several affected QoL domains, namely physical activity, strength impact, upper extremity, physical mobility, and a satisfaction in social roles [409]. Nevertheless, the application of general scales normally fails to detect impairments related to specificities of the disease (e.g., multi-system Involvement), which can lead in an overestimation of HrQoL. Regarding TNBC, HRQoL is also reduced, especially since patients experience distress because of the fear of demise or disease progression, self-image, and worry towards loved ones [410]. Besides, the HrQoL of women with TNBC is lower compared with women with other BC types [411].

PROMs that specifically assess HrQoL are a particularly relevant outcome in chronic and debilitating conditions, especially because biomarkers are not always associated with meaningful benefits to the patients [412]. Besides, the benefits of a possible lifesaving compared to possible serious, debilitating side effects should be careful considered. Therefore, tools that can accurately indicate how the patient feels and functions during the course of a disease or treatment indicate patient-valued outcomes that have influence in therapy approval. In the case of TNBC, several scales have been adopted to measure HrQoL, its response to treatments and side-effects [413].

However, in the case of PMM2-CDG, a specific and targeted HrQoL tool does not exist, which constitutes a challenge for current and future drug R&D programs and approval.

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CHAPTER 3.

MODEL OF INFLAMMATION: WHEN RARE GENETIC DISEASES GIVE YOU LEMONS, USE FIBROBLASTS

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Abstract

Background: Despite advances in diagnosing and treating genetic rare diseases (RD), research studies are challenging due to limited access to patients' tissues. Particularly, immune response research is hampered by the scarcity of cellular and animal models. Therefore, biobanks become important research tools, but are often limited to renewable and accessible cells, such as skin fibroblasts. Furthermore, fibroblasts' immune modulatory functions have been described. We aimed to describe the molecular response of human skin fibroblasts to inflammatory insults to validate them as a model for immunopathology studies, specifically in genetic RD.

Methods: Skin fibroblasts were stimulated with Tumour Necrosis Factor (TNF)-α and the transcriptome profiled by RNA-Seq. The genes differentially expressed between stimulated and non-stimulated cells were screened and used to predict cell-cell interactions. Functional enrichment analysis of transcription factors, pathways, and gene ontology biological processes was also performed. Data were validated by accessing the levels of proteins associated with the identified signalling pathways, by combining immunodetection assays.

Results: TNF-α caused transcriptional changes in fibroblasts similar to the response of immune cell lines. Based on the identified gene expression alterations, and putative receptor-ligand pairs, we also anticipated the interactions between fibroblasts and immune cells. Functional analysis revealed transcriptome alterations associated with immune-related pathways and processes, including TNF signalling (FDR=0.0002) and cell chemotaxis (FDR < 0.05). Additionally, we found an enrichment of nuclear factor kappa B (NF-κB) target genes (FDR < 0.05) and NF-kB activation, confirmed by the complete protein degradation of its inhibitor IκBa (p = 0.0019). Besides, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (FDR = 0.017) and p38 MAPK (FDR = 0.011) pathways were also activated. Lastly, we observed a significant induction of the secretion of interleukin 6 (p = 0.02), C-X-C motif chemokine ligand (CXCL) 8 (p = 0.027), C-C Motif chemokine ligand (CCL) 2 (p = 0.028) and CCL5 (p = 0.016).

Conclusion: This study contributes to the molecular characterization of the skin fibroblasts' response to TNF-α, revealing its intracellular pathways and secretome. It highlights the potential of fibroblasts as a reliable in *vitro* model for studying responses to inflammatory insults when other models are unavailable and provides an integrative platform transposable to any genetic RD that may provide novel clues for their diagnosis and treatment.

Keywords: Immune Response, Inflammation, Fibroblasts, Rare Diseases, transcriptome, Tumour Necrosis Factor-alpha.

3.1. Introduction

The immune response plays a crucial role in the pathogenesis and progression of many genetic rare diseases (RD) and the analysis of immune cells and biological processes in patient samples is essential to understand these diseases [1], [2]. Yet, among a myriad of challenges when studying these RD, tissue sample acquisition from patients is typically complex due to the small number of patients, and sampling process [3], [4]. In fact, tissue sampling requires the involvement of several stakeholders including different professional figures and patients themselves, as well as technology, institutional and ethical considerations to ensure patients are spared from unnecessary procedures and visits to health centres [5]. Nowadays, patient sample gathering and processing is primarily done through registries and specialized centres, making them rationally available to all in biobanks [6], [7]. For this reason, as shown by our multi-stakeholder community-centric approach, biobanks provide immense opportunities for RD communities, since they can mitigate sample access issues, and provide a source of disease models that can aid research and drug development [8], [9].

The most collected samples for biobanks are the most accessible ones, such as blood and biopsies. The resulting cell repositories typically contain cells which require little processing, are renewable and can provide a wealth of information about an individual's health status, such as primary skin fibroblasts [10]. The accessibility, the development of reprogramming approaches and evidence showing that fibroblasts provide a model system for many diseases, made fibroblasts a priceless primary resource, especially for RD research [10]–[13].

Fibroblasts are the main component of connective tissue [14], acquiring different morphological and functional differences depending on their specific location in the body [15]–[18]. This heterogeneity is reflected in unique molecular and structural profiles supporting vital functions, including trauma resistance, tissue repair, and reorganization of the extracellular matrix (ECM) [19], [20]. Additionally, they secrete several mediator proteins for local inflammation, such as cytokines and growth factors, allowing cell-cell communication with other cells involved in the immune system, such as T cells and macrophages [21]. Fibroblasts are ubiquitously found throughout the body and have been considered key sentinel cells for recognition of local internal and external pathological stimuli, contributing to immune response modulation to maintain homeostasis [22]. In fact, they have been reported to support and/or suppress immune responses related to infection [23], [24], autoimmunity [25], [26], and cancer [27].

Fibroblasts express several immune receptors, essential for the activation of a variety of signalling pathways [22], [28]. They express Toll-like receptors (TLR), which recognize pathogen-associated molecular patterns and trigger the immune response [29]; chemokine receptors, essential for immune cell migration to the injury site, proliferation and cytokine release modulation [30], [31]; growth factor receptors that are critical for cell development and tissue

repair [32]; and cytokine receptors which enable communication with the extracellular environment and induce intracellular biological responses [16]. Among these receptors are the tumour necrosis factor receptors (TNFR) 1 and 2 [33], which respond to the proinflammatory cytokine TNF-α and play a critical role in activating inflammatory, immune-modulatory, and apoptotic pathways [33], [34]. Activation of these receptors induces the production of several key interleukins (IL) and chemokines in fibroblasts, such as IL-6 and C-C Motif chemokine ligand (CCL) 2, which are involved in various biological roles, for example, leukocyte differentiation and recruitment [28], [35].

Fibroblast communication is critical for a fast and effective multicellular inflammatory response to aggression triggering several biological processes, namely cell migration, cell proliferation and differentiation, angiogenesis, and ECM rearrangement [36]. An ineffective and prolonged response can lead to chronic inflammation and lifelong consequences [37]. Activated fibroblasts producing uncontrolled immune regulators and ECM components can exacerbate chronic inflammation. Therefore, fibroblasts are essential in maintaining tissue homeostasis, regulating inflammatory response, and activating leukocytes [36], [38], [39].

The fibroblasts' features mentioned above suggest them as a potential in vitro model for studying the molecular mechanisms underlying immunopathology, particularly in cases where cellular and animal model organisms are scarce. Therefore, we mainly propose this model for RD with genetic aetiology due to their far-fetched single-centre sample collection. Particularly, for rare monogenic diseases with susceptibility to infection, like Phosphomannomutase 2-Congenital Disorder of Glycosylation, Coffin-Siris syndrome and periodontal Ehlers–Danlos syndromes, new models are not only beneficial but also extremely needed to the understanding of the immune defects [40]–[42].

With this pilot study, we aimed not only to molecularly describe the skin fibroblasts' response to TNF-α, but also to shed light on the potential of fibroblasts as a reliable *in vitro* model for studying inflammatory immune responses when other models are unavailable, particularly, in rare genetic diseases. Furthermore, we describe a methodology that answers a RD community need providing a platform to use biobank resources that can provide clues for understanding and treating RD.

3.2. Materials and Methods

3.2.1. Cells, cell culture and TNF-α stimulation

Three primary human skin fibroblasts lines derived from healthy donors from the same age range were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research - GM00498, GM00969 and GM03349. Cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) containing 1 g/L glucose and supplemented with L-glutamine (2mM), penicillin-streptavidin (100 units/mL and 100 µg/mL, respectively) and 10% (v/v) heat-inactivated foetal bovine serum - complete DMEM - at 37ºC in a 5% CO2 humidified incubator. All cell culture reagents were from Gibco. Regarding cell inoculation and stimulation condition, for the analysis of the gene expression and the transcriptome, 500 000 cells were seeded in T75 flasks and allowed to grow for 3 days. Cells were either stimulated with different concentrations or with 10 ng/ml of TNF-α during 5 h for IL-6 gene expression and transcriptome analysis, respectively. For the resazurin assay, fibroblasts were seeded in 96-well plates at 30 000 cells/ 200 µL/ well in triplicates. Cells were allowed to adhere for 24 h and stimulated with 200 µL of ten-fold serial dilutions of TNF-α-containing complete DMEM. The medium and the cells were recovered, analysed, or stored until further analysis. To detect the expression of signalling proteins by western blotting, 200 000 cells were seeded in 6 well plates and stimulated with 10 ng/ml of TNF-α from 0 to 120 min or for 30 min. An overview of the experimental design of this study is shown in Figure 3.1.

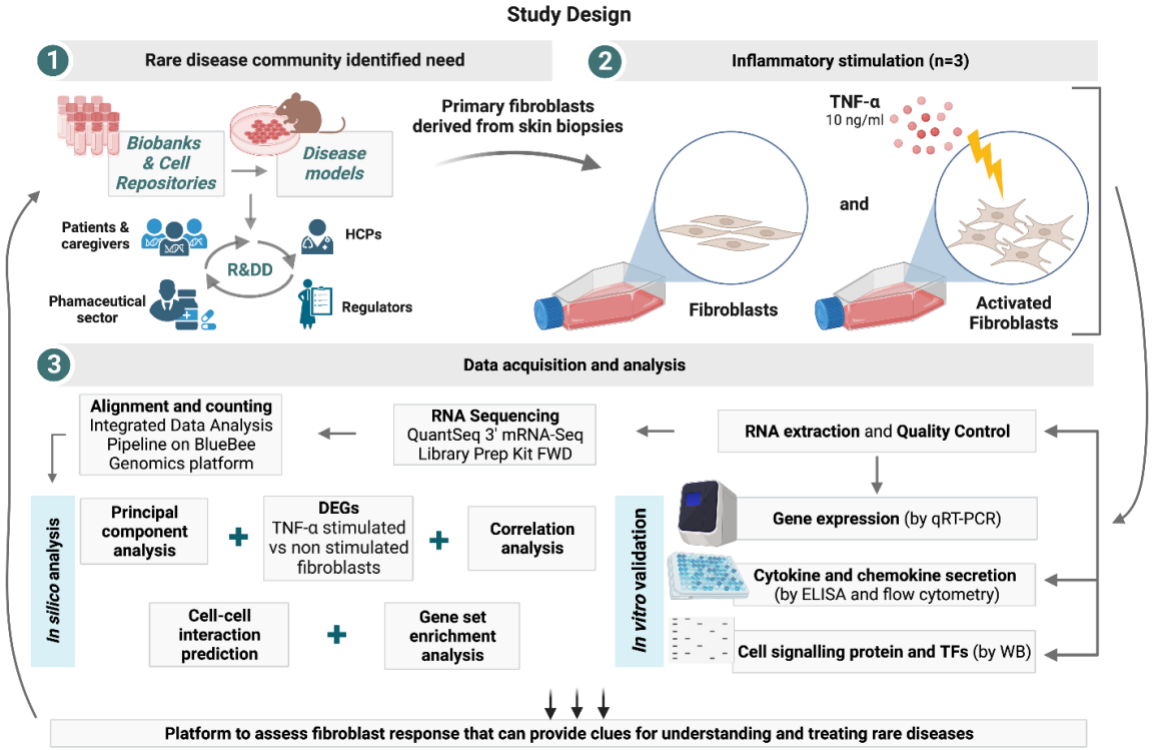

Figure 3.1. Overview of the study design. Schematic representation of the preclinical research study design aimed at characterizing skin fibroblasts as a potential in vitro model to study the immune responses to inflammatory insults

when other models are lacking, providing a platform to aid the understanding and treatment of rare diseases. Created with BioRender. Legend: DEGs – Differential expressed genes; HCPs – Healthcare professionals; TFs – Transcription factors; TNF– Tumour necrosis factor; WB – Western blot.

3.2.2. Cell viability and metabolic activity by the resazurin assay

The cell viability and metabolic activity were evaluated by analysing the conversion of resazurin to resorufin, as described [43]. After cell stimulation, the medium was replaced with 200 µL of complete DMEM containing 44 mM of resazurin and incubated for 3 h and 30 min. The 600 and 570 nm absorbances were measured in a UV-Vis microplate reader (SpectraMax190, Molecular Devices). Resorufin levels were estimated by calculating the difference between the absorbance values measured at 600 nm and 570 nm. The results of the stimulated samples were normalized against the non-stimulated control results.

3.2.3. RNA extraction and quality control

Total RNA from fibroblasts was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich) following manufacturer's instructions. RNA purity and concentration were accessed using NanoDrop (Thermo Fisher Scientific). RNA integrity analysis was performed resorting to the High Sensitivity RNA Analysis kit on Fragment Analyzer (Agilent Technologies Inc, USA). RNA was deemed acceptable if the 280:260 nm, 260:230 nm rations were higher than 1.9 and 1.5, respectively, and if the 28S:18S ration was higher than 2.0.

3.2.4. RNA sequencing (RNA-seq)

cDNAlibraries were prepared by the Genomics Unit of Instituto Gulbenkian de Ciência (Oeiras, Portugal) using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen Ghmb). RNA sequencing was performed on the NextSeq500 (Illumina) in a 75-base single-end mode, with a minimum target coverage of 6 million reads per library.

3.2.5. Transcriptome data analysis

3.2.5.1. Differential gene expression and correlation analyses of TNF-α stimulated fibroblasts

The QuantSeq 3' mRNA-Seq Integrated Data Analysis Pipeline from Lexogen GmbH on BlueBee® Genomics Platform was used to obtain the read counts for each gene of each sample.

Specifically, the quality control of the RNA-Seq raw data was assessed using the FASTQC (v 0.11.5) [44]. Then, the BBDuk software (v 35.92) was used to trim and remove the standard adapter sequences and poly(A) tails [45]. The resulting trimmed reads were aligned with the Genome Reference Consortium human build 38 (GRCh38) reference genome using STAR (v 2.5.2a) and counted with HTSeq [46], [47].

The transcriptomic statistical analysis was conducted using the R (v 4.1.1) computer language as well as most graphical representations, including principal component (PC) analysis, boxplot, heatmap, and volcano plots [48]. The scripts developed to conduct the analysis are available at: https://github.com/PGranjo/Model-of-inflammation.git. The quality-controlled read counts were assembled on a matrix and filtered of the low number read counts on each sample, using the "edgeR" (v 3.36) package [49]. The "edgeR'' package was also used to screen the differentially expressed genes (DEG) between TNF-α stimulated and non-stimulated cells using False Discovery Rate (FDR) $\leq$ 0.05 as cut-off.

To explore similarities in gene expression shifting patterns among fibroblasts and various immune cells in response to TNF-α, we resorted to publicly available data in the CytoSig database [50]. Specifically, RNA-Seq transcriptomic profiles from dermal fibroblasts and immune cells upon TNF-α stimulus regardless of the dosage were downloaded (data accession numbers: E-MTAB-5622 [51], GSE109460 [52], GSE100382 [53], GSE95588 [54], GSE129210 [55], GSE98624 [56], GSE40548 [57] and GSE70068 [58]). The parametric Pearson correlation was employed to examine the similarity of the transcriptome alterations upon TNF-α stimulation (log_2 Fold Change (FC)) between immune cells and skin fibroblasts, using FDR $\leq$ 0.05 as cut-off. To display the correlation results, the corrplot package (v 0.92) was utilized [59].

Gene expression analysis by real time - quantitative polymerase chain reaction (RT-qPCR)

3.2.5.2. Gene set enrichment analysis and cell-cell interactions analysis of TNF-α stimulated fibroblasts

Based on the ranked gene list using sign(log2(FC)) - log10(FDR) as a metric, a gene set enrichment analysis (GSEA) was carried out with the WEB-based GEne SeT AnaLysis Toolkit 2019 [60] with FDR ≤ 0.05 set as the cut-off criterion for all analyses as well as a minimum of 5 and maximum 2000 genes per category. This method was used to acquire enriched targets of transcription factors, Reactome pathways, and gene ontology (GO) biological processes non redundant terms (which are the most general categories in each branch of the GO Directed Acyclic Graph with the number of annotated genes ranging from 20 to 500). The enriched GO terms were further visualized through Cytoscape's Enrichment Map Plugin [61], [62]. For each group, the

broad terms with links with more than 5 nodes were selected using the WordCloud Plugin and encompassed by a circle [63].

To examine potential interactions between fibroblast and immune cells upon TNF-α stimulus, we utilized data on receptor-ligand pairs; and ligand and receptor repertoires of 144 primary cell types from supplementary materials 2 and 4 of a recent article related to the FANTOM 5 project [64]. The reported cell-cell interactions which relate to receptors and ligands that responded differentially to the stimuli in our samples were selected. The corresponding ligand/receptor pairs were analysed regarding their gene expression in different immune cell lines (Supplementary File 3.1). A threshold of 10 transcripts per million (~3 transcript copies per cell) was considered to identify expressed receptors and ligands in immune cells, as previously described [64], [65]. This enabled the creation of a network of potential interactions between our stimulated fibroblasts and several immune cells following TNF-α stimulus.

3.2.6. Gene expression analysis by real time - quantitative polymerase chain reaction (RT-qPCR)

RNA was converted to cDNA resorting to the High-Capacity cDNA Transcription Kit (Applied Biosystems) as described [66]. For the RT-qPCR, TaqMan chemistry was used, employing TaqMan probes and primers (Thermo Fisher Scientific) namely: IL-6 (Hs00174131_m1) and as the endogenous controls β-actin (4352935E) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 4333764F). The reaction was carried out on the Rotor-Gene 6000 Series (Corbett Research Ltd., UK). Gene expression was assessed by adapting the $2^{-\Delta\Delta}$ cycle threshold (CT) method [67]. Briefly, the ΔCT is first calculated which is the difference in the CT values for the gene of interest and the endogenous control genes, in a particular condition. The ΔΔCT is then calculated to compare the stimulated versus the non-stimulated condition by subtracting the non-stimulated ΔCT to the stimulated ΔCT.

3.2.7. Cytokine and chemokine secretion quantification by the ELISA and LegendPlex technologies

Cell supernatants were analysed by sandwich ELISA to determine the concentration of the cytokines IL-1β, IL-4, IL-6, IL12 (p40), IL-15, and IFN-y using commercial kits following the manufacturer's instructions (Immunotools). To quantify the developed signal, the 450nm absorbance was measured in a UV-Vis microplate reader (SpectraMax190, Molecular Devices). For the quantification of secreted chemokines, a Mix and Match panel based on the LEGENDplex™ Human Proinflammatory Chemokine Panel 1 (BioLegend) kit targeting C-X-C motif chemokine ligand (CXCL) 1, CXCL5, CXCL8, CCL2 and CCL5 was utilized, following the manufacturer's

instructions [68]. Signal quantification was performed in the BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences), according to the instructions. Cytokine and chemokine concentration was calculated using the specific standard curves. The concentration of both cytokines and chemokines was normalized to the protein concentration following the lysis and quantification of the cultured cells using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

3.2.8. Cell signalling proteins detection by western blot

Following stimulation, the cells were immediately washed with cold phosphate buffer saline and lysed in Pierce IP lysis buffer (Thermo Fisher Scientific) supplemented with protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche) and phosphatase (1 mM of sodium orthovanadate, Sigma-Aldrich) inhibitors. After the removal of cell debris, protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. For the detection of nuclear factor kappa B (NF-κB) p65, 25 μg of protein were separated on a 12.5 % SDS-Page acrylamide gel (Bio-Rad) during 4 h at 20 mA/gel and transferred for 2.5 h at 500 mA to PVDF membranes (Millipore). For the detection of the remaining proteins, 30 μg of protein extract were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membranes were blocked by either Carbofree blocking solution (VectorLabs) or 5% non-fat dried milk. Immunoblotting was carried out with antibodies against NF-κB p65 (1:500, sc-372), Iκ-Bα (1:600, sc-371), p38 (1:1000, sc-728) (Santa Cruz Biotechnology), P-p38 (1:1000, #9211), extracellular signal-regulated kinase 1/2 (ERK1/2, 1:1000, #9102) and P-ERK1/2 (1:1000, #9101) (Cell Signaling) overnight, followed by 1 h incubation with the Peroxidase AffiniPure donkey anti-rabbit IgG (H+L) (1:10000, #711-035-152, Jackson Laboratories) or anti-mouse-IgG-HRP (1:2500, #554002, BD Biosciences) secondary antibodies. Signal was revealed using the Lumi-Light Western Blotting Substrate (Roche) to X-ray films (Amersham Hyperfilm™ ECL). For loading control, the membranes were stripped subsequently using ReStore® Western Blot stripping buffer (Thermo Fisher Scientific) following the manufacturer's instructions and then re-probed using a mouse monoclonal anti-α-tubulin antibody (T6074, 1:50000, Sigma-Aldrich). The optical density of bands was quantitated using the ImageJ v.1.43 software and normalized using the housekeeping protein normalization method, based on the fact that these proteins are ubiquitously and constitutively expressed within cells and tissues [69].

3.2.9. Statistical analysis

The statistical analysis performed on transcriptome data is described under the Transcriptome Data Analysis section of the methods. When multiple testing was performed, p-values were adjusted using the FDR method. The remaining data are presented as mean ±

standard deviation, and statistical significance was analysed using GraphPad Prism (v.8.4.0, GraphPad LLC). Data normality was accessed by the Shapiro-Wilk test. Comparisons of means between two groups were analysed using a two-tailed unpaired Student's t-test. Comparisons of means between three or more groups were analysed using the one-way ANOVA with Dunnett's multiple comparison tests. Differences were considered statistically significant if p-value/FDR $\leq$ 0.05 or marginally significant if 0.05 < p-value/FDR $\leq$ 0.1.

3.3. Results

3.3.1. TNF-α stimulation in skin fibroblasts mimics transcriptome alterations observed in immune cell lines

To assess the response of human skin fibroblasts to an inflammatory stimulus, we subjected the cells to escalating doses of TNF-α, a major acute pro-inflammatory cytokine. The cells' response was evaluated in terms of the expression of *IL6*, the metabolic activity and viability. Our results indicate that TNF-α stimulus with several dosages induces the gene expression of *IL6* in skin fibroblasts, with higher doses tending to lead to increasing gene expression of *IL6* (Figure 3.2A). Additionally, TNF-α treatment leads to a 10 to 15% increase in the cell metabolic activity without viability loss (Figure 3.2B, Supplementary Figure 3.1). The fibroblasts used showed expression of both TNF-α receptors, with higher expression of the TNFR1 when compared to TNFR2 (Supplementary Figure 3.2).

To further investigate the response upon TNF-α stimuli, we analysed the overall transcriptomic profile of the fibroblasts, which showed a consistent gene expression distribution between cell lines (Supplementary Figure 3.3). Following RNA sequencing, a PC analysis shows that TNF-α stimulated and non-stimulated samples cluster separately, indicating that all stimulated samples respond to TNF-α in the same direction (as indicated by the PC1 explaining 46.17 % of sample variance) and that the two groups of samples have distinct transcription profiles (Figure 3.2C). We proceeded to explore the differential gene expression between both conditions and identified 159 DEG. Specifically, 130 upregulated and 29 downregulated DEG were identified in the stimulated condition in comparison to the non-stimulated condition (Figure 3.2D). The list of DEG and respective FDR values can be found in Supplementary File 2).

We next interrogated if the fibroblast response mimics the response of other cells, with particular focus on the cells involved in the immune response. This was possible resorting to the CytoSig database which contains transcriptomic profiles of different cell lines in response different stimulus, including TNF-α. Therefore, we explored the correlation of the transcriptomic alterations of our TNF-α-stimulated fibroblasts to available profiles of immune cells (namely, T cells,

monocytes, macrophages, and astrocytes) and other dermal fibroblasts. Correlations with other dermal fibroblasts (r = 0.51, FDR ≤ 0.05), macrophages (r = 0.32, FDR ≤ 0.05), neutrophils (r = 0.30 and r = 0.29, FDR ≤ 0.05) and monocytes (r = 0.28 and r = 0.24, FDR ≤ 0.05) as well as with T cells (r = 0.17, FDR ≤ 0.05 and r = 0.14, FDR ≤ 0.05 for $CD4^+$ memory T and Jurkat T cells cells, respectively) were observed. Our results show that the response of our stimulated fibroblasts not only significantly and positively correlates with the one from dermal fibroblasts, but also with the response to TNF-α of immune effector cells, including neutrophils, monocytes, macrophages, and T cells (Figure 3.2E).

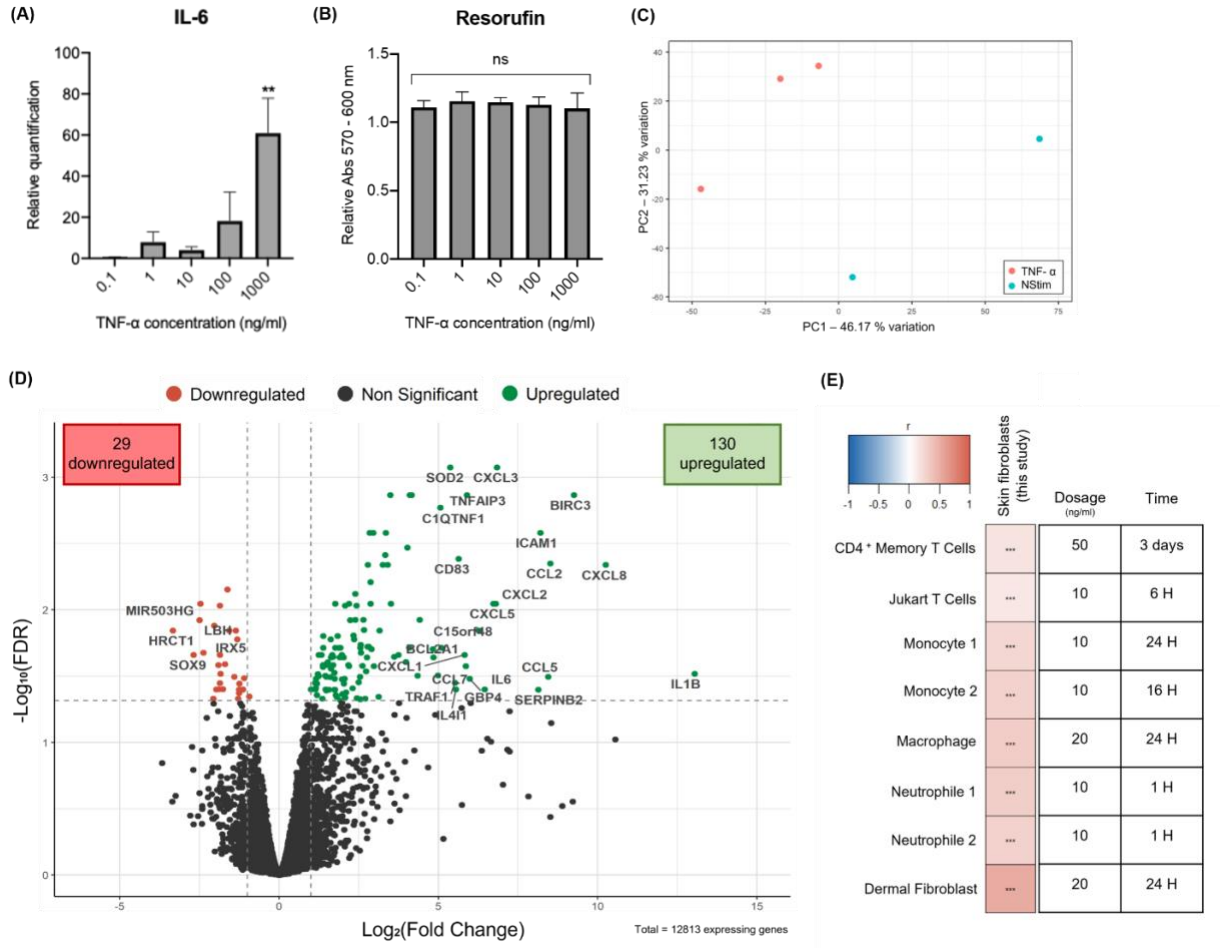

Figure 3.2. Tumour Necrosis Factor (TNF)-α causes a shift in the fibroblasts' transcriptional profile which correlated with the immune cells' response. (A) Relative IL6 gene expression in response to TNF-α stimulation (t = 5 h) at various concentrations (0.1, 1, 10, 100 and 1000 ng/ml) quantified by RT-qPCR. IL6 mRNA levels were normalised using β-actin and GAPDH as endogenous controls and the relative quantification is related to the non-stimulated condition ($2^{-\Delta\Delta CT}$ method). Data are represented as the mean ± SD (n = 3). Statistically significant values were accessed by a one-way ANOVA with Dunnett's multiple comparisons (False Discovery Rate (FDR) < 0.01 (**)); (B) Cell viability and measurement of metabolic activity of fibroblasts after TNF-α stimulus (t = 24 h) at different concentrations (0.1, 1, 10, 100 and 1000 ng/ml) quantified by the resazurin assay. Data are represented as the mean ± SD (n = 3). Relative values are related to the non-stimulated condition. Values between different concentration are not significantly different as indicated by a one-way ANOVA with Dunnett's multiple comparisons; (C) Principal component (PC) analysis biplots of

human skin fibroblasts before and upon TNF-α stimulus (10 ng/ml, t = 5 h). Stimulated (red dots, n = 3) and non-stimulated (NStim, blue dots, n = 2) samples are distinguished by two factors: PC1 and PC2, which account for 46.17% and 31.23% of the data variability, respectively. The non-stimulated condition of one of the samples was excluded from this analysis due to an RNA sequencing technical issue; (D) Volcano plot of all expressing genes. Differential expressed genes (DEG) cut of criteria was of an FDR < 0.05. Red dots represent the downregulated DEG, green dots represent the upregulated genes and black dots represent all the genes that are not differentially expressed upon stimulation (FDR > 0.05); (E) The whole transcriptomic differential expression profile of fibroblasts upon stimulus have a positive correlation with all TNF-α-stimulated immune cell transcriptomic profiles in the CytoSig database (FDR < 0.001 (***) based on parametric Pearson correlation).

3.3.2. Fibroblast's response to TNF-α activates signalling pathways congruent with predicted fibroblast-immune cell interactions

Following the analysis of the expression profiles of fibroblasts stimulated or not with TNF-α, a GSEA was performed to assess the enriched targets of transcription factors, pathways, and biological processes. The lists of all the gene ontology terms and respective FDR values can be found in Supplementary File 3.3. The top enriched transcription factors' targets altered after TNF-α mainly hint for the activation of the NF-κB pathway (Figure 3.3A, Supplementary Table 3.1). Indeed, 7 different NF-κB transcription factor binding motifs (M1-M7) constitute the top of this list. Other transcription factor targets include binding motifs of interferon regulatory factors, signal transducer and activator of transcription family members, and the nuclear factor of activated T-cells. Moreover, a total of 150 pathway-related gene sets were significantly enriched following stimulation with TNF-α, including mainly various cytokine and chemokine signalling pathways as well as TNF receptor and transforming growth factor b-activated kinase 1 (TAK1)-induced activation of NF-κB signalling, which agrees with the identified transcription factors (Figure 3.3B).

Regarding the enriched biological processes, our results further support the above-mentioned observations associating the cytokine/chemokine signalling to important immune response-related processes, namely chemotaxis, leukocyte adhesion, IL-6 production, and the regulation of the inflammatory response (Figure 3.3C). Importantly, a large overlap was observed between the enriched biological processes (Figure 3.3D, Supplementary File 3.3), where the main clusters correspond to cytokine production and lymphocyte activation, directly linked with the regulation of protein localization and secretion, the regulation of the inflammatory response, chemotaxis, and cell migration as well as response to interferon. Other clusters include: 1) the regulation of the endoplasmic reticulum - including terms related to a series of enzymatic reactions (e.g., proteolysis and hydrolysis necessary for several biological processes, for instance, intracellular and ER-nucleus signalling) as well as the export of proteins to the cytoplasm and subsequent exocytosis; 2) apoptotic signalling and 3) negative regulation of stimulus (Figure 3.3D).

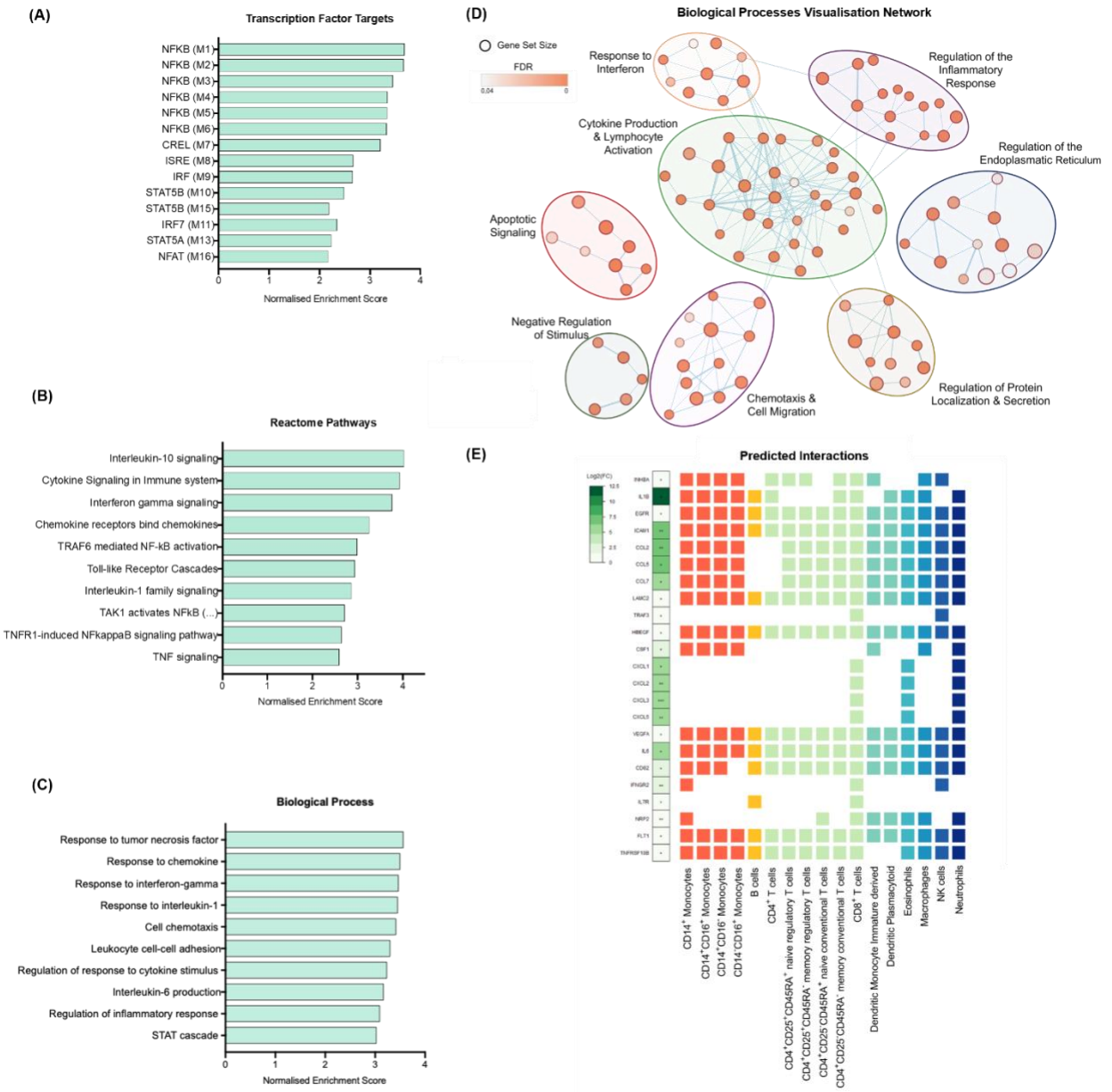

Figure 3.3. Gene Set Enrichment Analysis (GSEA) plots of patient-derived fibroblasts in response to Tumour Necrosis Factor (TNF)-α (t = 5 h) to identify enriched transcription factors, pathways, and biological process, and fibroblasts-immune cell predicted interactions based on differential expressed genes (DEG). (A) Normalized enrichment score (NES) of the top statistically significant enriched transcription factors' targets. Motifs for which the transcription factor is not annotated (unknown) are not represented but can be found in Supplementary material 2 – Supplementary Table 1; (B) NES from immune-related pathways (from Reactome). The complete list of pathways is found at Supplementary material 4; (C) NES from non-redundant inflammatory related biological process terms. All GSEA analysis were performed using the WebGestalt platform and all displayed terms are statistically significant (False Discovery Rate (FDR) < 0.05); (D) Enrichment map of TNF-α stimulation on skin fibroblasts. The visualization network displays the enriched biological processes gene-sets of stimulated fibroblasts. The size of nodes characterizes the number of genes inserted in each data set and the orange gradient represents the significance of each term. Light orange is less statistically significant than strong orange. Clusters of functionally related gene-sets were manually circled and labelled, with the help of the WordCloud Plugin. Links with fewer than 5 nodes were excluded; (E) Log2 Fold-Change (FC) of differently expressed receptors and ligands upon TNF-α stimulus in skin fibroblasts, annotated with a prediction of cell–

cell interactions with immune cells. These Log2FC are statistically significant, with FDR < 0.05 (*), FDR < 0.01 (**) or FDR < 0.001 (***).

Based on the identified functions, we interrogated the possible cell-cell interactions of the TNF-α stimulated fibroblasts and immune cells. Figure 3.3E shows the changes in expression of the DEG that codify receptors and ligands in the fibroblasts and the possible crosstalk with their pairs present in immune cells based on annotated literature. As shown in Figure 3.3E, the upregulation of *ICAM1* may potentiate the interaction between skin fibroblasts and all the immune cells represented (macrophages, T cells, monocytes, B cells, natural killer (NK) cells, dendritic cells, neutrophils, and eosinophils), since all of them express one or more receptors known to ligate ICAM1 (i.e., *ITGB2, ITGAL, ITGAX, ITGAM, IL2RA* and *ILR2G*) (Supplementary File 3.1). The upregulation of *CCL2, CCL5* and *CCL7* in stimulated fibroblasts might mean an increased expression of these chemokines and, therefore, influence the recruitment of immune cells expressing corresponding receptors (macrophages, naive and memory regulatory/conventional T cells, monocytes, NK cells, dendritic cells, neutrophils, and eosinophils). On the other hand, *CXCL1, CXCL2, CXCL3* and *CXCL5* gene products can potentially attract cytotoxic $CD8^+$ T cell, eosinophils, and neutrophils, according to their CCR/CXCR expression.

3.3.3. Experimental validation of the TNF-induced pathways through NF-κB and MAPK signalling activation

To experimentally validate the intracellular signalling induced by TNF-α, we first examined the gene expression associated with the above identified pathways and processes, namely cytokines, chemokines, and signalling proteins or transcription factors. According to Figure 3.4A, genes that codify cytokines with roles in the initiation of the acute phase response and triggering of the adaptive immune responses upregulate their expression upon TNF-α stimulation, namely *IL1B*, *IL6* and *IL15*. Other transcripts related to well described pro-inflammatory and anti-inflammatory cytokines were not detected (i.e., *IL4*, *IL10*, *IL12* and *IFNG*). Also upregulated on stimulated fibroblasts are the *CXCL1*, *CXCL5*, *CXCL8*, *CCL2* and *CCL5* chemokine genes. Other proteins, specifically transcription factors, namely, *NFKB1, NFKB2 and NFKBIA,* also have their gene expression increased following the inflammatory trigger (Figure 3.4A).

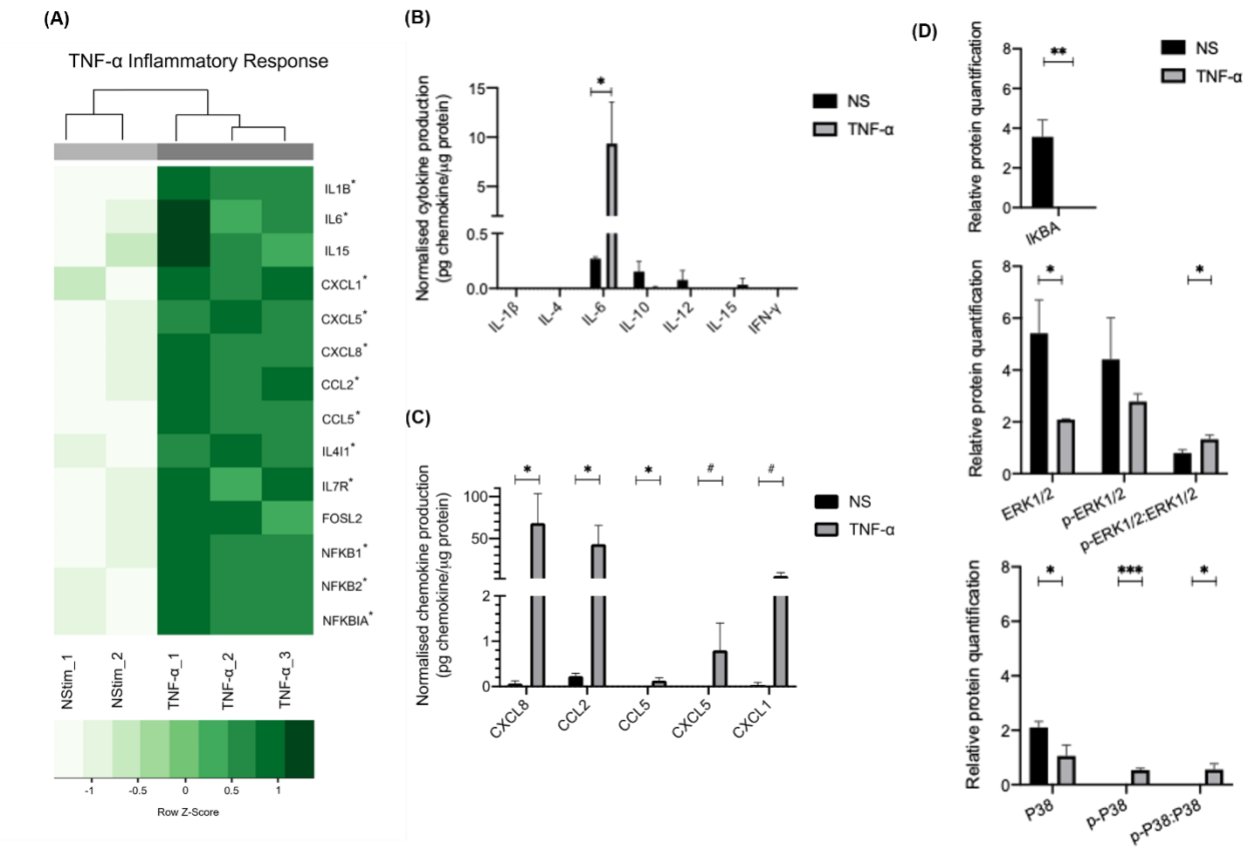

Figure 3.4. Gene and protein expression of Tumour Necrosis Factor (TNF)-α downstream signalling proteins and analysis of inducible cytokines. (A) Heatmap of immune-related gene expression of non-stimulated (NS) (light grey) and TNF-α stimulated (dark grey) fibroblasts (t = 5 h). The green gradient represents the expression of each gene of each sample presented as a z-score, with lighter green indicating lower levels of gene expression and darker green indicating higher levels of gene expression. Genes marked with '*' are differently expressed (False Discovery Rate (FDR) < 0.05); (B) Cytokine production of NS and TNF-α stimulated samples (t = 24 h) measured by ELISA. Data are represented as the mean ± SD (n = 3). Statistically significant values were accessed by two-tailed unpaired Student's t-tests ($p < 0.05$ (*)). Normalized values were obtained by dividing the concentration of cytokines (pg/ml) by the concentration of total protein (Ug/ml); (C) Chemokine production of NS and TNF-α stimulated samples (t = 24 h) quantified using a Mix and Match LegendPlex kit. Data are represented as the mean ± SD (n = 3). Statistically significant values were accessed by two-tailed unpaired Student's t-tests ($p < 0.05$ (*) and $0.1 < p < 0.05$ (#)). Normalised values were obtained by dividing the concentration of chemokines(pg/ml) by the concentration of total protein (µg/ml); (D) Relative protein quantification of cell signalling proteins and transcription factors (namely I*k*Bα, p38, p-p38, extracellular signal-regulated kinase (ERK)1/2 and p-ERK1/2) of NS and TNF-α stimulated fibroblasts (t = 30 min) samples by western blot. The detection of α-tubulin was used for loading control and for band normalization (housekeeping protein normalization method). Statistically significant values were accessed by two-tailed unpaired Student's t-tests for I*k*Bα ($p < 0.01$ (**) and by the one-way ANOVA with Dunnett's multiple comparisons for the ERK1/2, p-ERK1/2, p38, p-p38 and respective ratios (FDR < 0.05 (*), FDR < 0.001 (***)).

At the protein level, we could observe significant expression of IL-6 ($p = 0.02$) but not IL-1β and IL-15 (Figure 3.4B). Due to the lack of detectable gene transcripts, as expected, protein secretion of IL-4, IL-10, IL-12 and IFN-γ was also not detected. The secretory profile of fibroblasts consisted mostly of chemokines (Figure 3.4C). In fact, the pro-inflammatory chemokines C-X-C

motif chemokine 8 (CXCL8, p = 0.027), C-C motif chemokine 2 (CCL2, p = 0.028) and CCL5 (p = 0.016) were found to be significantly overexpressed upon TNF-α stimulation. In addition, CXCL5 and CXCL1 protein expressions were found to be marginally significantly overexpressed (p = 0.055 and p = 0.082, respectively). These increase in cytokines and chemokines was consistent between all cell lines and validate the above-mentioned gene expression alterations (Supplementary Table 3.2).

We proceeded for the analysis of signalling proteins involved in the TNF-α receptors' downstream signalling, namely the NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways. Considering the high enrichment of NF-κB targets, we analysed the protein expression of its p65 subunit. The expression of this transcription factor did not seem to change with increasing stimulation times from 0 to 120 minutes (Supplementary Figure 3.4). However, we simultaneously analysed the expression of its endogenous inhibitor IκBα whose expression is completely abrogated upon TNF-α stimulation (p = 0.0019, Figure 3.4D, Supplementary Figure 3.5), pointing towards the activation of the NF-κB pathway. The analysis of the other downstream proteins tested also showed significant differences upon TNF-α stimulation, namely the ERK1/2 (FDR = 0.017), p38 (FDR = 0.011) and p-p38 (FDR = 0.0003) proteins (Figure 3.4D, Supplementary Figure 3.5)). Further analysis also points to the activation of both the MAPK/ERK and p38 MAPK pathways, since the p-ERK1/2:ERK1/2 and p-p38:p38 ratios significantly increase upon TNF-α stimulation (FDR = 0.017 and FDR = 0.011, respectively).

All in all, our data show that TNF-α activates several pathways in skin fibroblasts, namely the NF-κB MAPK/ERK and p38 MAPK signalling pathways which, individually or collectively, are responsible for increased IL-6 and chemokine expression.

3.4. Discussion

New European regulations restrict the use of animal models in research prioritizing the combination of *in vitro* testing of cellular models with computational analysis as a reliable alternative to address immune physiology and pathology [70]. The scenario is even more delicate for RD as there is a general lack of *in vivo* and *in vitro* models and difficulty of getting patient samples [71]. As such, initiatives such as the as the EuroBioBank, the Telethon Network of Genetic Biobanks and the NIGMS Human Genetic Cell Repository are critical to aid basic and translational research, maximizing outcomes with limited resources [7], [72], [73].

Fibroblasts' cultures were previously widely used for biochemical studies before genetic testing was available. This allowed the biobanking of these primary cells which provided easy to use and genetically stable models with high *in vivo* relevance [74]. They have been used to study immunopathology in the context of autoimmune and inflammatory conditions like psoriasis,

rheumatoid arthritis, Chron's disease, and many others [75]–[77]. Since cells within the body share mechanisms and biological processes which can be switched on or off, depending on the milieu and immediate body requirements, our hypothesis that fibroblasts can be used to study immune defects gains relevance, especially in the context of rare genetic diseases that affect multiple cell types or body systems, given that pathogenic variants may, in principle, affect molecular mechanisms systemically regardless of cell types or tissue. Therefore, for these diseases, especially when the mechanisms of action are unknown, skin fibroblasts can be a useful model for study the disease pathogenesis.

We selected the inflammatory TNF-α stimulus to characterize how skin fibroblasts respond to inflammatory stimuli at the molecular and functional level. It was previously reported that TNF-α causes transcriptional changes related to the cytokine signalling which are in part controlled by anti-TNF-α therapy in a time-dependent manner [78]. However, to the best of our knowledge, this is the first study resorting to RNA sequencing to deeply analyse the transcriptome profile in response to this cytokine. Badran *et al* performed RNA sequencing of TNF-α-stimulated dermal fibroblasts to analyse gene expression differences in fibroblasts from patients with *RELA* haploinsufficiency [79]. However, the fibroblast response to this cytokine was not fully described, only focusing on the pathogenic alterations in diseased fibroblasts. Nevertheless, the response to TNF-α we describe is significantly and positively correlated with the one described in the above-mentioned study as well as with other stimulated immune cell lines, regardless of the TNF-α dosage, type of RNA-sequencing and stimulation time (Figure 2E). This finding highlights the immunological role of fibroblasts, as they activate similar pathways to those of immune cells. Next research efforts should aim to describe exactly which pathways are similarly activated between fibroblasts and each immune cell type specifically to fully portrait their potential as immune cell models.

Our functional analysis of the transcriptome confirmed the role of skin fibroblasts as supporters of an immune response. In fact, it showed the activation of immune-related pathways, the production of effector cytokines, their chemoattractant role, and the adhesion to leukocytes, which were previously reported to some extent [28]. This is consonant with the observed enrichment of several motifs of the NF-κB transcription factor, well known to be involved in these immune and inflammatory processes [80]. Additionally, the induction of genes related to apoptosis by TNF-α, was also detected, a response extensively described following the engagement of TNFR1 and his death domain [81]. Surprisingly, some transcription factors, pathways and biological processes concerning the TLR and interferon signalling were also enriched. Since TLR are receptors that are activated after the recognition of pathogen-associated molecular patterns derived from microbes, the enrichment of this pathway was not expected after the TNF-α challenge. However, it is well established that TNFR and TLR share downstream signal transduction mechanisms, mainly by the formation of TAK1-TAK1-binding proteins complexes

which lead to activation of NF-κB, c-Jun N-terminal kinase and p38 signalling [82]. Even though TLRs differ from TNFR in terms of the usage of intracellular adaptors, they ultimately lead to the downstream recruitment of TAK1-binding proteins to form the complex, leading to the activation of the same pathways as TNF-α [83]. Regarding the interferon signalling, it is likely that its enrichment derived from its counteracting and anti-inflammatory role against TNF-α, as required for the control and fine-tuning of the magnitude and extent of the inflammatory reaction, ensuring homeostasis and preventing a deleterious response [84]. Accordingly, two of the main biological processes' clusters, specifically, interferon signalling and regulation of inflammatory response, are associated (Figure 3D).

To validate some of the observations obtained by our *in silico* analysis, we conducted *in vitro* experiments, mainly focused on the production and secretion of cytokines and chemokines as well as the expression of signal transduction players and transcription factors. From the cytokines analysed after TNF-α stimulation, we could only detect the secretion of IL-6 in skin fibroblasts. The inducible production of IL-6 following several stimuli has been previously reported in dermal and other fibroblast types [85]–[88], as well as its pleiotropic functions, which include roles in innate and adaptive immune response but also cell proliferation or differentiation [89]. We could not detect secreted IL-1β nor IL-15 even though our data show that their gene expression is upregulated nearly 13- and 3-fold, respectively, when treated with TNF-α. IL-1β is produced in an inactive pro-form that needs to be cleaved by the caspase-1 inflammasome before cellular release and acquire biological activity [90]. It is possible that the mechanism that control this post translational modification of IL-1β pro-form and other mechanisms involved in its regulation and release depend on the duration and intensity of the stimuli [91], [92]. Concerning IL-15, it has been shown that very few cells secrete detectable levels of this protein due to the presence of many initiation codons in the 5' untranslated regions that compromise the efficiency of protein translation [93]. Therefore, it may be worth to explore IL-15 expression with different translation-oriented approaches.

Confirming the first-line warning role of fibroblasts as part of the innate immunity, we show the synthesis of five chemokines upon inflammatory stimulus, previously shown to be produced by skin fibroblasts following TNF-α or other biological or chemical challenges [94]–[98]. The expression of this chemokines together with IL-6 and the *in silico* observations previously discussed confirm the inflammatory response of the fibroblasts under study given their association with inflammatory diseases [12], [89], [99], [100]. Under inflammatory conditions, CXCL1, CXCL5 and CXCL8 have a main chemotactic influence on neutrophils, but attraction and migration of T cells and basophils has also been reported for CXCL8. Additionally, CCL5 attracts monocytes, eosinophils, and subsets of lymphocytes, while CCL2 regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells [101]. However, our predictive analysis of receptor-ligand interactions between fibroblasts and immune cells suggests that

several additional interactions may occur, beyond those mentioned above. Therefore, it would be interesting to demonstrate the specificity and functional impact of the stimulated skin fibroblasts response products towards a range of immune cells. As an example, the role of IL-6 (shown to potentially interact with all represented immune cells' subsets) is complex and context-dependent, leading to different functional outcomes depending on the target cell. Secreted IL-6 can (1) induce the differentiation and activation of immune cells, such as monocytes, T and B cells [102]–[104]; (2) stimulate antibody production by B cells [105] as well as (3) activate innate immune cells, such as macrophages and neutrophils, enhancing their phagocytosis and cytokine secretion [106], [107], among other functions. Even though these studies suggest that skin fibroblasts-produced IL-6 have important implications for skin immunity, it is important to note that the IL-6 function may be influenced by other cytokines and cells present in the skin microenvironment [103], [105]. Therefore, further research is needed to fully elucidate the mechanisms underlying skin fibroblasts-secreted IL-6 effects on different immune cells *in vitro* and *in vivo*. The analysis of cell signalling molecules and/or transcription factors confirmed the activation of the NF-κB, the MAPK/ERK and p38 MAPK signalling which have been previously described as TNF-α-induced downstream pathways [108], [109]. This is not surprising given their well described roles in inflammation-related processes [80], [110].

Our transcriptomic analysis provided a comprehensive description of fibroblasts' molecular response, which was confirmed *in vitro* by the overexpression of cytokines, chemokines as well as NF-κB transcription factor activation at the protein level upon TNF-α stimulus. Our methodology allowed us to comprehensibly describe the fibroblasts' response to TNF-α at the molecular and mechanistic level. Additionally, we answered our hypothesis by linking the response of skin fibroblasts to that of several immune cell lines, proposing them as cellular models to study immunological defects. The findings of this study might stimulate the use of cell biobanks, particularly, deposited skin fibroblasts, for research into certain disease areas, particularly rare genetic diseases, when the goal is studying immunopathology. This approach is further supported by the fact that fibroblasts are easily genetically modifiable cells [111], [112], if patient-derived skin fibroblasts are not available.

While innovative, our study presents some limitations, including a small sample size and limited depth of RNA sequencing. Additionally, the PC analysis shows some heterogeneity between the transcriptome of the different lines, with two of them (2 and 3 years old at biopsy) aligning closely in comparison to the third one (10 years old at biopsy). This is in line with the reported donor skin heterogeneity and dynamic alterations in cell populations with age [113] and explains, at least in part, the variability observed. Nevertheless, all fibroblasts responded consistently to TNF-α as shown by the 46,1% directional shift in the PC analysis, the 159 commonly and significantly altered genes and the consistent *in vitro* alteration of TNF-α-induced proteins. In sum, this is a pilot study to prove the concept of a new model to study immunopathology in

diseases where sample size is limited. In these circumstances, and providing that sample show little heterogeneity, sample size may be considered adequate to draw preliminary conclusions. In addition, further studies are required to validate and/ or describe the predicted interactions between stimulated fibroblasts and immune cells, as well as the subsequent functional implications. Furthermore, it has also been reported that skin fibroblasts respond to other pathogen- or danger- associated molecular patterns [114], [115] and, accordingly, we observed that they respond to lipopolysaccharide and phorbol 12-myristate 13-acetate by inducing *IL6* mRNA expression (data not shown). Therefore, this study could be complemented hereafter by the assessment of the fibroblasts' response and its similarity to the immune cells' response to other stimuli (e.g., other cytokines and pathogens).

3.5. Conclusion

This study answers to the RD community need and priority of increasing the use of RD biobanks and the available cell models to enlighten RD mechanisms and facilitate basic and translational research. Not only it is a further contribution to widening the knowledge on skin fibroblasts' immunity, specifically the inflammatory response to TNF-α but also it shows that even when conventional immune cells are not available, fibroblasts can be reliable models to study intra and intercellular immune mechanisms. In the field of rare genetic diseases, this contribution can reduce the impact of challenging sample access in immunopathology research.

3.6. References

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CHAPTER 4.

A LOOK INTO THE IMMUNOPATHOLOGY IN PMM2-CDG: HOW DOES DEFECTIVE GLYCOSYLATION IMPACT THE TNF-TNFR1 AXIS?

MANUSCRIPT UNDER PREPARATION

Abstract

Background: Phosphomannomutase 2-CDG (PMM2-CDG) the most predominant of a group of approximately 170 rare genetic diseases resulting from defects in glycosylation pathways – Congenital Disorders of Glycosylation (CDG). The ubiquitous role of glycosylation explain why CDG are frequently multi-systemic. Similarly, the fact that the immune system is frequently affected in CDG is unsurprising give the glycosylation importance for the immune system function. Specifically, PMM2-CDG patients experience recurrent, severe, and sometimes lethal infections, particularly during childhood. However, the mechanism behind immunological (dys)function of PMM2-CDG patients are still not elucidates.

Methods: We resorted to a comprehensive approach including transcriptomics and its validation through a set of experimental assays following the engagement of TNFR1 by TNF-α stimulation (10ng/ml) in a cohort of 3 PMM2-CDG and wild-type (WT) skin fibroblasts.

Results: PMM2-CDG cells showed a deficient N-glycosylation profile as well as TNFR1 shedding following TNF-α stimulation. Besides, RNA sequencing and transcriptomic analysis showed that fibroblasts bearing the R141H heterozygous variant have a different transcriptional profile which translate in differences in several immune-related and signalling processes, highlighted by functional enrichment analysis. Validation of the transcriptomic analysis results show that TNF-α stimulated PMM2-CDG fibroblasts significantly and marginally significantly secrete less IL-6 (p = 0.03) and CCL5 (p = 0.07) cytokines, respectively, than control fibroblast, important mediators of inflammation and infection. Additionally, contrastingly to WT samples, ERK1/2, p38 and JNK2 signalling molecules are not significantly overexpressed in PMM2-CDG cells following stimulation. This results a significantly lower expression of p38 (p = 0.003) in PMM2-CDG samples.

Conclusion: This methodology allowed to identify defects in biological pathways that might be behind immunological dysfunction in PMM2-CDG, particularly focusing on TNFR1 signalling, consequently highlighting potential therapeutic targets for PMM2-CDG. Furthermore, it provided molecular insights that might be relevant for clinical manifestations related with other systems (e.g., liver fibrosis).

Keywords: Tumour Necrosis Factor-alpha, Fibroblasts, PMM2-CDG, Inflammation, Transcriptome

4.1. Introduction

Immunity is the capacity of the body to resist to disease-causing agents, like harmful pathogens and cancer-related cell abnormalities. It is operated by a complex network of cells, molecules and mechanisms that dynamically cooperate in either a general and immediate manner – innate response – or a more gradual and specific way – adaptive response [1]. A fully operational and correct action of the immune system relies on a series of recognition, interaction, activation, and communication events between adjacent or remote cells. These processes are dependent on and highly modulated by oligosaccharide structures that decorate practically all immune receptors and effectors – the glycans [2].

Glycans are synthetized and attached to proteins and lipids by glycosyltransferases and glycosidases through a process called glycosylation. The resulting repertoire of glycans – the glycome – reflects not only the several glycosylation types but also the overall expression of glycan-modifying enzymes and their substrate availability [3]–[5]. The glycome exert vital structural and functional roles in their glycoconjugates [6], [7]. The essential function of glycosylation on the organism, including in the immune system, is well demonstrated when it goes wrong.

Congenital disorders of glycosylation (CDG) are a group of approximately 170 inborn errors of metabolism caused by genetic variants in proteins involved in any step of glycosylation, resulting in defective glycophenotypes [8]. Due to vast and crucial biological roles of glycans, CDG often present a myriad of clinical manifestations that can be limited to a single organ but are most often multi-systemic [9]. While the neurological system is known to be the most predominantly affected leading to very prevalent development delays and intellectual disability, immunological abnormalities are a hallmark of twelve CDG [10], [11]. Nonetheless, corroborating the important role of glycosylation for the immune system function, many other CDG frequently present immune clinical signs, particularly, phosphomannomutase 2 (PMM2)-CDG – the most frequent CDG [12].

PMM2-CDG was the first-ever described CDG in 1980 and, since then, more than 900 patients have been reported worldwide [13], [14]. It is a multi-system disease with autosomal recessive inheritance mostly affecting the neurological, ophthalmologic, coagulation and gastrointestinal systems [14]. Individuals with PMM2-CDG often present recurrent and severe infections, which are not only relevant from a clinical but also patient-reported perspective. On the one hand, infections are the cause for many hospitalizations, therapeutic interventions, and fatalities [15], [16]. Besides, they can also trigger other severe clinical manifestations, like seizure and stroke-like episodes [17], [18]. On the other hand, families consider infectious episodes as one of the most impactful PMM2-CDG-associated symptoms, which significantly impairs their quality of life [16], [19]. Some PMM2-CDG patients can also present cellular and biochemical

alterations. Increased white blood counts, increased or decreased natural killer (NK) cells, T cell lymphopenia, neutropenia, and hypogammaglobulinemia are among the clinical findings [20]–[25]. An inflammatory response with high concentrations of inflammatory cytokines in serum as well as in abdominal and pericardial fluids was also reported in PMM2-CDG patients [26]. However, mechanistically, little is known about the immune dysfunction in PMM2-CDG. Limited reports indicate decreased neutrophil chemotaxis and increased NK cell reactivity [22]. Besides, hypoglycosylation of membranal proteins in PMM2-CDG patients-derived cells was shown to decrease their binding to respective antigens [27].

We hypothesize that defective glycosylation of immune receptors disrupts intracellular signalling causing mechanistic faults behind immune dysregulation in PMM2-CDG. Some of the most well studied glycosylated receptors are the tumour necrosis factor (TNF) receptor 1 and 2 (TNFR1/2). These two receptors differ in terms of structure, number of glycosylation sites (four and six, respectively), expression pattern, use of adaptor proteins and signalling mechanisms as well as specificity to different forms of TNF-α. While TNFR1 is more specific for and activated by the soluble form, TNFR2 is more specific and can only be fully activated by TNF-α membrane-bound forms [28]. For this reason, we will focus our study in the TNFR1 downstream signalling. The binding of TNF to TNFR1 sequentially recruits of tumour necrosis factor receptor type 1-associated death domain (TRADD) protein, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), TNF receptor associated factor 2 (TRAF2) or TRAF5, and cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2. TRAF proteins and cIAP1/2 are ubiquitin E3 enzymes that add ubiquitin chains to RIPK1. Ubiquitinated RIPK1 recruits TGFβ-activated kinase 1 (TAK1) -binding protein 2 (TAB2) and TAB3, which then activate NF-κB, JUN N-terminal kinase (JNK) and p38 signalling [29], [30]. TRAF2 is also thought to activate a mitogen-activated protein kinase kinase kinase (MAPKKK), such as apoptosis-stimulated kinase 1 which leads to the activation of not only JNK but also ERK [31]. In alternative to these pathways, when RIPK1 is not ubiquitylated, TNF-driven apoptotic signalling mediated by FAS-associated death domain (FADD) protein, caspase 8 (CASP8) and regulated by CASP8 and FADD-like apoptosis regulator (also called cFLIP). Alternatively, degradation or depletion of cIAPs reduces or prevents RIPK1 ubiquitylation promoting its assembling with RIPK3, pro-caspase 8 and cFLIP which also induces apoptosis. Finally, if RIPK1 is deubiquitylated but caspases are inactivated, necroptosis takes place [29]. How exactly defective glycosylation can affect the above described TNFR1 signalling is still fully elucidated.

Therefore, in this study, we aimed to investigate the effect of deficient glycosylation in the TNF/TNFR1 axis in PMM2-CDG patients-derived fibroblasts using a transcriptomics approach. Furthermore, our enrichment analysis and experimental validation help elucidating the affected processes and pathways in PMM2-CDG. Concluding, our work contributes to the understanding

of defective pathways behind immune dysfunction in PMM2-CDG highlighting potential immunological-tailored therapeutic targets.

4.2. Materials and Methods

4.2.1. Fibroblasts acquisition, culture and stimulation

Skin fibroblasts derived from PMM2-CDG and apparently healthy individuals were acquired from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. Cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) with 1 g/L glucose with 2 mM L-glutamine, penicillin-streptavidin (100 units/mL and 100 µg/mL, respectively) and 10% (v/v) heat-inactivated foetal bovine serum (FBS), all from Gibco, at 37ºC in a 5% CO2 humidified incubator.

Seeding of 5 x 10^5 fibroblasts was performed in T75 flasks, allowed to grow for 72 hours and stimulated 10 ng/ml of TNF-α for 5 hours (for RNA sequencing) or for 24 hours (for lectin staining, membranal protein detection or protein detection from whole lysates). For signalling proteins and secreted protein expression assays, 2 x 10^5 fibroblasts were seeded in 6 well-plates, allowed to grow for 3 days and stimulated for 30 minutes or for 24 hours, respectively. After stimulation, the medium and the cells were collected, centrifuged, and immediately used or stored at -20ºC until further analysis.

For a clear picture of the experimental assays carried on with the cells, the study design is depicted in Figure 4.1.

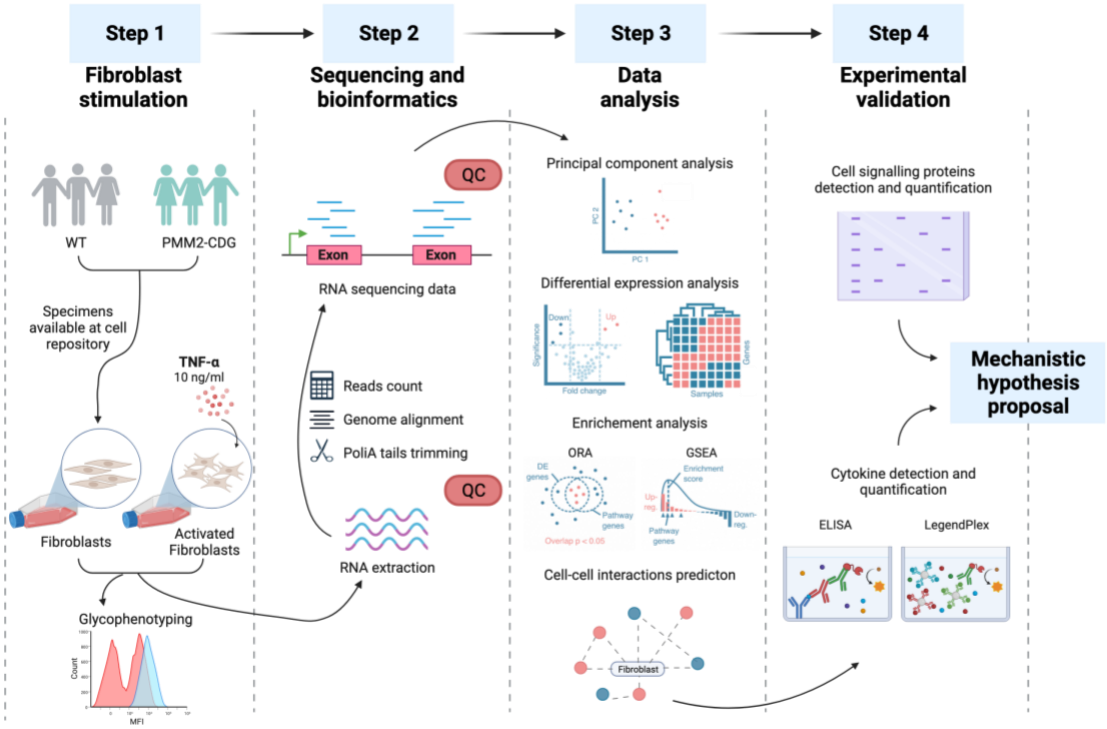

Figure 4.1. Study design.

4.2.2. Cell phenotyping by flow cytometry

To analyse the overall cell N-glycosylation, 1 x 10^5 cells were stained with a 1:100 dilution of concanavalin A (ConA)-biotin (#B-1005) and Galanthus nivalis (GNL)-biotin (#B-1245), from VectorLabs at 4 °C for 20 min. Streptavidin-PE (#554061, BD Pharmingen) at a 1:100 dilution was used for secondary detection. For cell surface TNFR1 staining, the PE anti-human CD120a antibody was used in a 1:100 dilution (#369903, Biolegend) at 4 °C for 20 min. After staining, cells were washed and fixed with 2% paraformaldehyde. Data were acquired using the Attune Acoustic Focusing Cytometer (Applied Biosystems, Waltham, MA, USA) and analysed with FlowJo software version 10.0.5 (BD Biosciences, Franklin Lakes, NJ, USA). Data were presented as delta mean fluorescent intensity (MFI) obtained by subtracting the MFI of the secondary staining control or unstained cells, as appropriate.

4.2.3. RNA extraction and quality control

Fibroblasts' total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich), following the manufacturer's instructions. RNA purity and concentration were accessed using NanoDrop (Thermo Fisher Scientific) and RNA integrity was analysed using the High Sensitivity RNA Analysis kit on Fragment Analyzer (Agilent Technologies Inc). RNA was

deemed acceptable if the 280:260 nm, 260:230 nm rations were higher than 1.9 and 1.5, respectively, and if the 28S:18S ration was higher than 2.0.

4.2.4. RNA Sequencing (RNA-seq) and alignment

cDNA libraries were prepared by the Genomics Unit of Instituto Gulbenkian de Ciência (Oeiras, Portugal) using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Ghmb). RNA sequencing was performed on the NextSeq500 (Illumina) in a 75-base single-end mode, with a minimum target coverage of 6 million reads per library.

The QuantSeq 3′ mRNA-Seq Integrated Data Analysis Pipeline (Lexogen GmbH) on BlueBee® Genomics Platform was employed to obtain the read counts for each sample. Specifically, the RNA-seq raw data quality control was assessed using the FASTQC (v 0.11.5) [32]. Then, the BBDuk software (v 35.92) was used to trim and remove the standard adapter sequences and poly(A) tails [33]. Lastly, the reads were aligned with the reference genome (GRCh38) and counted using STAR (v 2.5.2a) and HTSeq, respectively [34], [35].

Individual-level data was submitted at the database of Genotypes and Phenotypes (dbGaP) and are available for download by authorized investigators. Public summary-level phenotype data are available at https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs003313.v1.p1.

4.2.5. Differential gene expression and functional enrichment of WT and PMM2-CDG TNF-α-stimulated fibroblasts

Transcriptomic analysis and most graphical representations including principal component analysis (PCA), volcano plots, scatter plot, functional enrichment plot, heatmaps and interaction plot were performed using the R (v 4.1.1) computer language [32]. The scripts developed for the conduction of this analysis are available at: https://github.com/PGranjo/Immunological-Involvement-in-PMM2-CDG.git. The quality controlled read counts were assembled on a matrix and the low quality read counts were filtered. The differentially expressed genes (DEG) between TNF-α stimulated and non-stimulated conditions of the PMM2-CDG and WT samples were obtained using the "edgeR" (v 3.36) package with a cut-off of False Discovery Rate (FDR) ≤ 0.05).

An over-representation analysis was performed to obtain the enriched pathways and gene ontology biological processes upon TNF-α stimulation using the Toppfun functionality of the Toppgene Suite (v 31) platform [33]. FDR adjusted p ≤ 0.05 was set as the cut-off criteria. The fold enrichment was calculated manually using a contingency matrix. In this matrix, the enrichment was derived from the formula: (number of DEG present in the term × numbers of genes not

present in the term from the reference genomes) ÷ (number of DEG not present in the term × number of genes from the reference genome present in the term). The enriched gene ontology terms were further visualised on Reactome [34].

4.2.6. Predicted cell-cell interactions between TNF-α stimulated fibroblasts and immune cells

Based on receptor-ligand pairs and ligand and receptor repertoires of 144 primary cell types from supplementary materials 2 and 4 of a recent article related to the FANTOM 5 project (64), we examined potential interactions between fibroblast and immune cells upon TNF-α stimulus First, we selected cell-cell interactions associated with receptors or extracellular ligands codified by the identified DEG. Then, we assessed the gene expression of the corresponding ligand/receptor pairs in different immune cell lines, with a threshold of 10 transcripts per million (~3 transcript copies per cell), as previously described (Supplementary File 4.1) (64,65). The output was a list of potential interactions between our stimulated fibroblasts and several immune cells following TNF-α stimulus.

4.2.7. Quantification of secreted cytokines using ELISA and LEGENDplex™

The concentration of the cytokines IL-1β, IL-4, IL-6, IL12 (p40), IL-15, and IFN-y was determined by sandwich ELISA using development kits according to the manufacturer's (Immunotools) instructions. Signal was quantified by the measurement of the absorbance at 450 nm on a microplate reader (SpectraMax 190 Microplate Reader, Molecular Devices. A Mix and Match panel based on the LEGENDplex™ Human Proinflammatory Chemokine Panel 1 kit (BioLegend) was used following the manufacturer's instructions to measure the following chemokines: CXCL1, CXCL5, CXCL8, C-C Motif chemokine ligand (CCL) 2 and CCL5. Signal detection was performed in the BD LSRFortessaTM X-20 Cell Analyzer (BD Biosciences), according to the instructions. Both cytokine and chemokine concentrations were obtained using the specific standard curves and normalized to the total protein concentration following the lysis and quantification of the cultured cells using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

4.2.8. Detection and quantification of PMM2, TNFR1 and signalling proteins by Western blot

Following stimulation, the cells were immediately washed with cold phosphate buffer saline and lysed in Pierce IP lysis buffer (Thermo Fisher Scientific) supplemented with protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche) and, in the case of lysates for signalling proteins detection, phosphatase (1 mM of sodium orthovanadate, Sigma-Aldrich) inhibitors. After the removal of cell debris, protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. 30 µg of protein extract were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat dried milk and immunoblotting was carried out with antibodies against PMM2 (1:1000, Proteintech), TNFR1 (H-5) (1:500, sc-8436), p38 (1:1000, sc-728) (Santa Cruz Biotechnology), extracellular signal-regulated kinase 1/2 (ERK1/2, 1:1000, #9102) and JNK2 (1:1000, # #9258) (Cell Signaling) overnight, followed by 1 h incubation with the Peroxidase AffiniPure donkey anti-rabbit IgG (H+L) (1:10000, #711-035-152, Jackson Laboratories) or anti-mouse-IgG-HRP (1:2500, #554002, BD Biosciences) secondary antibodies. Signal was revealed using the Lumi-Light Western Blotting Substrate (Roche) to X-ray films (Amersham HyperfilmTM ECL). For loading control, the membranes were stripped subsequently using ReStore® Western Blot stripping buffer (Thermo Fisher Scientific) following the manufacturer's instructions and then re-probed using a mouse monoclonal anti-α-tubulin antibody (T6074, 1:50000, Sigma-Aldrich). The optical density of bands was quantitated using the ImageJ v.1.43 software and normalized using the housekeeping protein normalization method, since these proteins are ubiquitously and constitutively expressed within cells and tissues (69).

4.2.9. Statistical analysis

The statistical analysis performed on transcriptome data is described under the Differential gene expression and functional enrichment of WT and PMM2-CDG TNF-α-stimulated fibroblasts section of the methods. For the remaining data, statistical significance was analysed using GraphPad Prism (v.8.4.0, GraphPad LLC). Data normality was accessed by the Shapiro-Wilk test. Comparisons of means between the four sample groups were analysed using the one-way ANOVA with Dunnett's multiple comparison tests. p-values were adjusted using the FDR method. Differences were considered statistically significant if p-value/FDR ≤ 0.05 or marginally significant if 0.05 < p-value/FDR ≤ 0.1.

4.3. Results

4.3.1. PMM2-CDG fibroblasts are characterized by changes in the normal N-glycophenotype which are significant upon TNF-α stimulation

Previous studies have suggested that PMM2-CDG patients have a differential inflammatory response which could contribute to the burden of the disease [35], [36]. Here, we studied skin fibroblasts from 3 PMM2-CDG patients and 3 controls to better understand the pathological mechanisms. As represented in Table 4.1, the average age for PMM2-CDG was 4.3 years old, and 5 years old for controls. All patients were heterozygous, and the most frequently reported mutation in the literature, p.R141H, was shared by two patients [37]. All patients showed reduced expression of PMM2 protein (Supplementary Figure 4.1).

Table 4.1. Clinical data of the PMM2-CDG patients and healthy individuals included in this study.

Study ID	Coriell ID	Mutated gene	Gene variants	Gender	Age at sampling (years)
P1	GM20945	PMM2	c.95TA>GC/c.470T>C (p.L32R/p.F157S)	Female	7
P2	GM27226	PMM2	c.422G>A/c.647A>T (p.R141H/p.N216I)	Male	1
P3	GM27386	PMM2	c.422G>A/c.415G>A (p.R141H/p.E139K)	Female	5
C1	GM00498	-	-	Male	3
C2	GM00969	-	-	Female	2
C3	GM03349	-	-	Male	10

PMM2-CDG is often associated with abnormal glycosylation profiles [38], [39]. Moreover, inflammation has been associated with glycosylation alterations which vary depending on the cell type and environment [40], [41]. Therefore, we accessed the glycoprofiles of the cohort under study and how it is affected by the soluble TNF-α pro-inflammatory stimulus. To do this, we quantified the binding of two lectins with specificity to N-glycan-related structures, namely ConA and GNL [42]. Results show that PMM2-CDG fibroblasts show variable and non-significant N-glycosylation alterations under normal conditions (Figure 4.2A). However, upon TNF-α stimulation, it seems that PMM2-CDG fibroblasts present less high-mannose terminated glycans

(Man3-Man9) and early terminated biantennary N glycans, as shown by ConA staining (p = 0.009), and decreased alpha1-6 and alpha 1-3 mannose-terminated N-glycans, considering GNL staining (p = 0.008). Next, we assessed the surface and overall expression of TNFR1 in our cohort of skin fibroblasts. Figure 4.2B shows that TNFR1 expression decreases after TNF-α stimulation and that there are no differences between the surface expression of TNFR1 between WT and PMM2-CDG fibroblasts, before or after stimulation. Furthermore, the analysis of TNFR1 expression in whole lysates from one PMM2-CDG patient and one controls: (1) shows that TNF-α stimulation seems to increase TNFR1 glycosylation in WT and PMM2-CDG fibroblasts (as per the appearance of a >55 kDa band in the stimulated condition) and (2) highlighted the presence of a 34 kDa TNFR1 fragment in the PMM2-CDG stimulated sample (Figure 4.2C). This result point to the possibility that even though the overall TNFR1 glycosylation in PMM2-CDG might be similar to the control samples, surface N-glycosylation is impaired and seems to structurally impact the TNFR1 response after TNFR1 engagement by TNF-α stimulation.

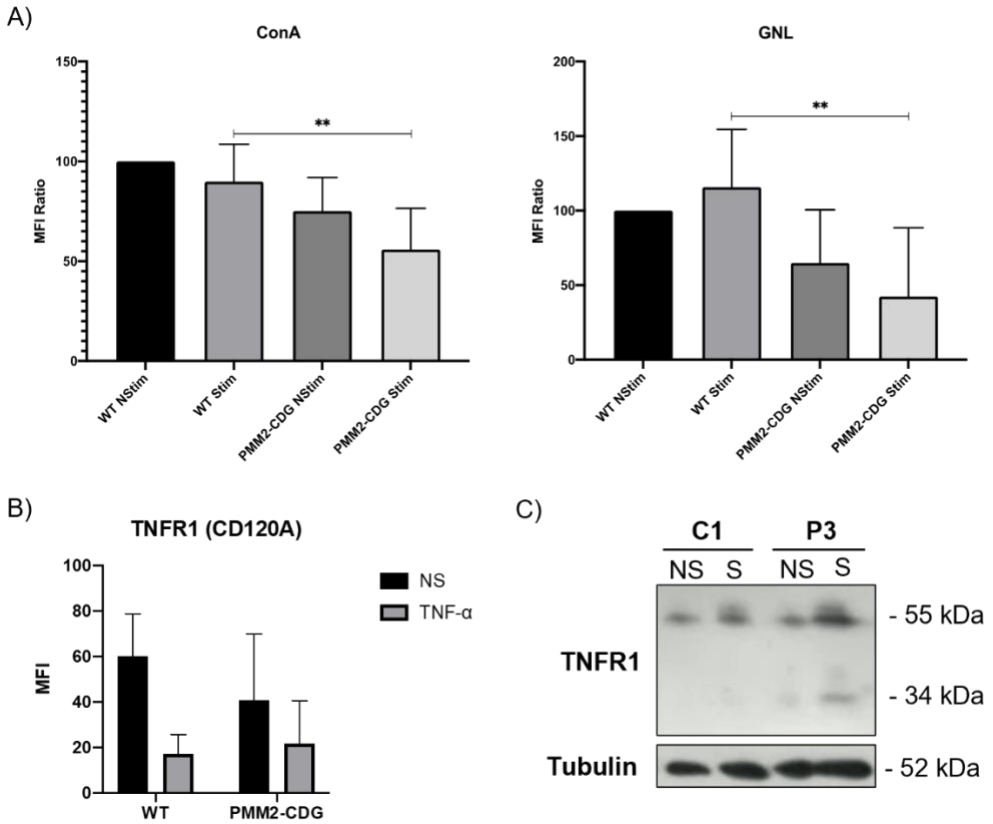

Figure 4.2. Glycoprofiling and cell phenotyping in stimulated and non-stimulated. PMM2-CDG and WT fibroblasts. Fibroblasts were stimulated with 10 ng/ml of TNF-α for 24h and analysed using different techniques. (A) Lectin staining was performed resorting to the ConA and GNL lectins. Data are represented as the mean ± SD of normalized MFI values to the WT NStim group (n = 3).; (B) flow cytometry was performed for TNFR1 cell surface staining and (C) western blot of cell lysates was performed for TNFR1 immunoblotting to access the overall expression of TNFR1.

Staining with α-tubulin was as loading control. Statistically significant values were accessed by a one-way ANOVA with Dunnett's multiple comparisons ($p < 0.01$ (**)).

4.3.2. PMM2-CDG fibroblasts have a different gene transcription profile than WT samples with and without a proinflammatory stimulus, which is genetic variant-dependent

To analyse the transcriptome profile of fibroblasts in a steady state and after inflammatory stimuli, we applied mRNA-Seq in fibroblasts stimulated or not with TNF-α. A principal component (PC) analysis clustered samples in four separate sample groups (Figure 4.3A). The PC1 accounted for 25.82 % of sample variation and enabled us to discern between the stimulated and non-stimulated samples. The PC2 separated most of our PMM2-CDG and WT samples, with 20.32 % sample dispersion. Nonetheless, non-stimulated and stimulated samples from the PMM2-CDG patient with p.L32R/p.F157S genotype aggregated with WT samples from each respective condition (Figure 4.3A), suggesting a transcriptome most similar to WT than the other PMM2-CDG genotypes analysed in this study. The PC analysis considering patients with the most frequent and severe genetic variant p.R141H (p.R141H/p.N216I and p.R141H/p.E139K genotypes) and controls resulted in a clearer data variability explanation, suggesting that this variant has bigger impact in the cellular transcriptional profile (Figure 4.3B).

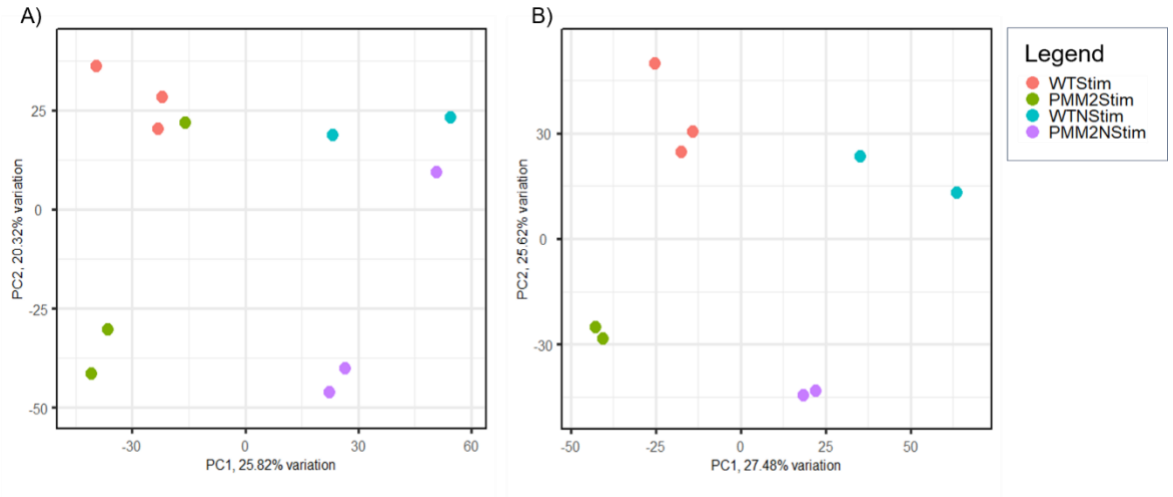

Figure 4.3. Principal component (PC) analysis. PCA biplots of WT and PMM2-CDG samples before and upon TNF-α stimulus of (A) all WT and PMM2-CDG stimulated and non-stimulated samples and (B) of WT and PMM2-CDG samples bearing the heterozygous genetic variant p.R141H (GM 27226 and GM 27386). Legend: PC – principal component.

4.3.3. Genes related to inflammatory response are differentially expressed in PMM2-CDG fibroblasts

To better understand the difference between the transcriptome alterations of TNF-α-stimulated WT and p.R141H-bearing PMM2-CDG samples, we analysed the differential expressed genes (DEG) that significantly respond to TNF-α for each condition. We identified 305 and 222 DEG in the WT and PMM2-CDG cells upon TNF-α stimulus, respectively (Figure 4.4, Supplementary File 4.2). Further examination revealed that 239 of the 305 DEG from the WT group are upregulated and 66 are downregulated. In contrast, the PMM2-CDG sample group encompasses 188 upregulated and 34 downregulated genes. When comparing the DEG from the WT and PMM2-CDG conditions, it can be noted that they fall into three distinct categories: Common, WT-exclusive, and PMM2-CDG-exclusive DEG (Figure 4.4B). Specifically focusing on the Common group, 183 DEG are shared between the PMM2-CDG and WT samples in response to TNF-α stimulation, from which 159 are upregulated and 24 are downregulated. Among the upregulated common DEG, we found genes that codify proteins with immune-related functions, namely, *ICAM1*, *IL32*, *IL4I1*, *IL1B*, *CXCL8*, *TNFAIP3*, *NFKB2*, *NFKB1*, *NFKBIA*, *NFKBIE*, *NFKBIB*, *NFKBIZ*, and *RELB*. Nevertheless, some of these common genes between WT and PMM2-CDG still respond differently to the stimulus. This is the case of *IL1B* and *CXCL8* genes which, after stimuli, display a clear expression difference between WT (log2FC > 10, Figure 4.4A) and PMM2-CDG (log2FC < 10, Figure 4.4A) after TNF-α stimulation. The WT-exclusive group consists of 122 DEG, 80 of which are upregulated, while 42 are downregulated following TNF-α stimulation. Some interleukin-related genes, such as *IL6*, *IL34*, *IL15*, and *IL7R*, are among the upregulated DEG. Finally, the PMM2-CDG-exclusive group contains 39 DEG, 29 upregulated and 10 downregulated. *RELA*, an NF-κB subunit, is an upregulated gene in this group, whereas *FOS*, *RUNX1T1*, *ZFHX4*, *SOX12*, and *ID1* are among the downregulated genes (Figure 4.4B).

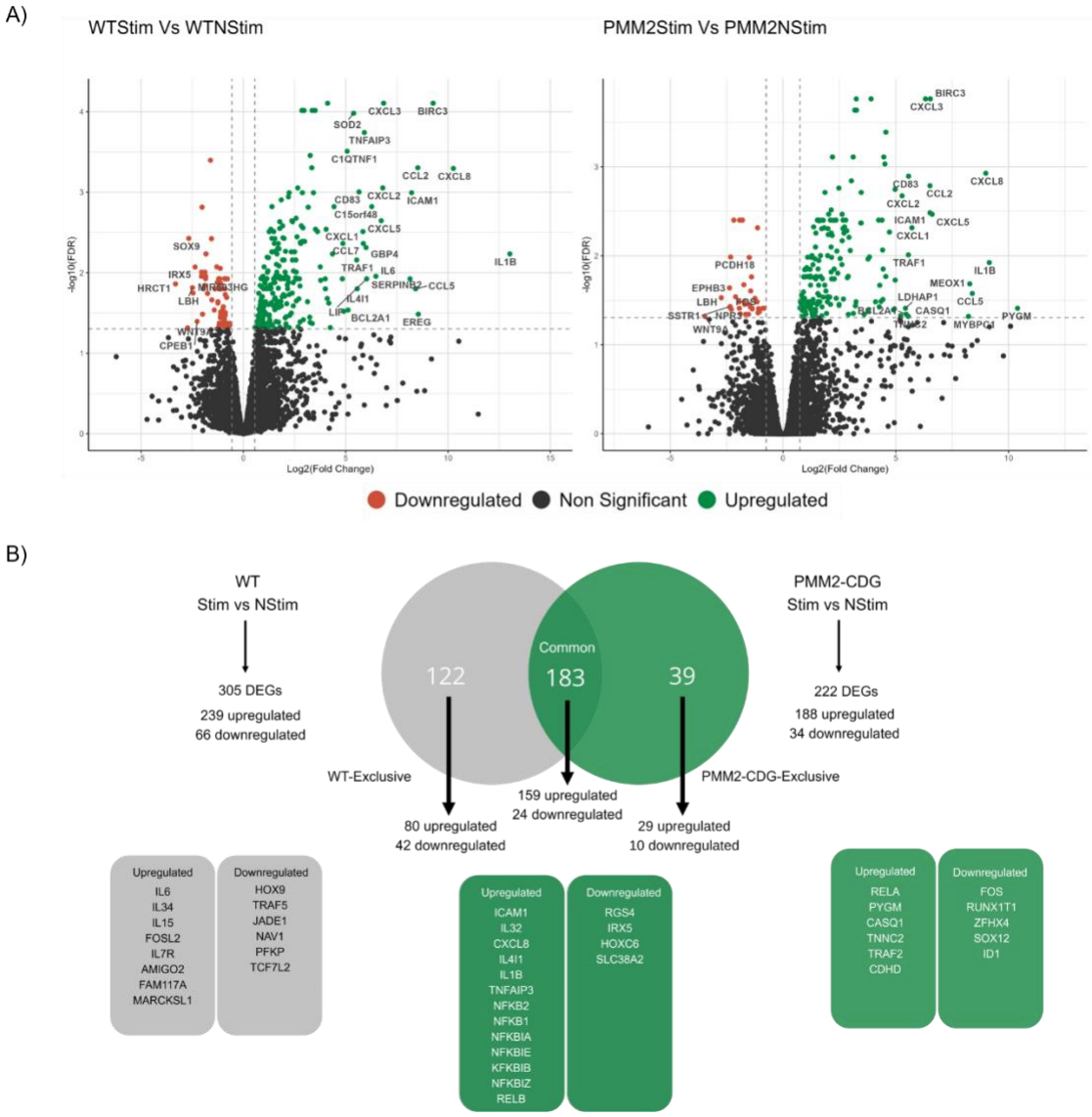

Figure 4.4. Differential gene expression between TNF-α stimulated and non-stimulates samples of WT and PMM2-CDG samples. (A) Volcano plot of the differential expressed genes (DEG) of WT fibroblasts (left) and PMM2-CDG (right) fibroblasts upon TNF-α stimulus. The cut of criteria to select the DEG was an adj. P-value < 0.05. Red dots represent all the DEG (P-value < 0.05 and log2FC > 0.56); black dots represent all the genes with a log2FC > 0.56 but that are not significantly different before and after stimulation (adj. P-value > 0.05); lastly, grey dots comprise all non-differentially expressed genes with a log2FC < 0.56. The 0.56 of Log2FC was selected due to being the lowest log2FC in both groups. (B) Venn diagram of WT vs PMM2-CDG differentially expressed genes (DEG) upon TNF-α stimulus. A total of 305 (239 upregulated and 66 downregulated) and 222 DEG (188 upregulated and 34 downregulated) were identified in the WT and PMM2-CDG sample groups, respectively. These DEG can be classified into three distinct groups: WT-exclusive, Common, and PMM2-CDG-exclusive. There is a total of 122 DEG in the WT-exclusive group, of which 80 are upregulated (e.g., IL6, IL34, IL15, FOSL2, IL7R, AMIGO2, FAM117A, and MARCKSL1) and 42 are downregulated (i.e., HOX9, TRAF5, JADE1, NAV1, PFKP, TCFL2). The Common group contains 183 DEG, 159 upregulated (such as ICAM1, IL32, CXCL8, IL4I1, IL1B, TNFAIP3, NFKB2, NFKB1, NFKBIA, NFBIE, NFKBIB, NFKBIZ, RELB) and 24 downregulated (e.g., RGS4, IRX5, HOXC6, SLC38A2). The PMM2-CDG-exclusive group comprises 39 DEG, of which 29 are upregulated (including RELA, PYGM, CASQ1, TNNC2, TRAF2, and CDHD) and 10 are downregulated (for instance, FOS, RUNX1T1, ZFHX4, SOX12, and ID1).

4.3.4. In-depth functional annotation analysis upon TNF-α stimulus

The identified DEG were functionally annotated, and the enriched gene ontology (GO) terms were categorized into three groups: Common GO terms, WT-exclusive GO terms, and PMM2-CDG-exclusive GO terms (Supplementary Files 4.3, 4.4 and 4.5, respectively). Their counts and distribution across GO categories according to their associated genes' up- or downregulation is depicted in Figure 4.5A.

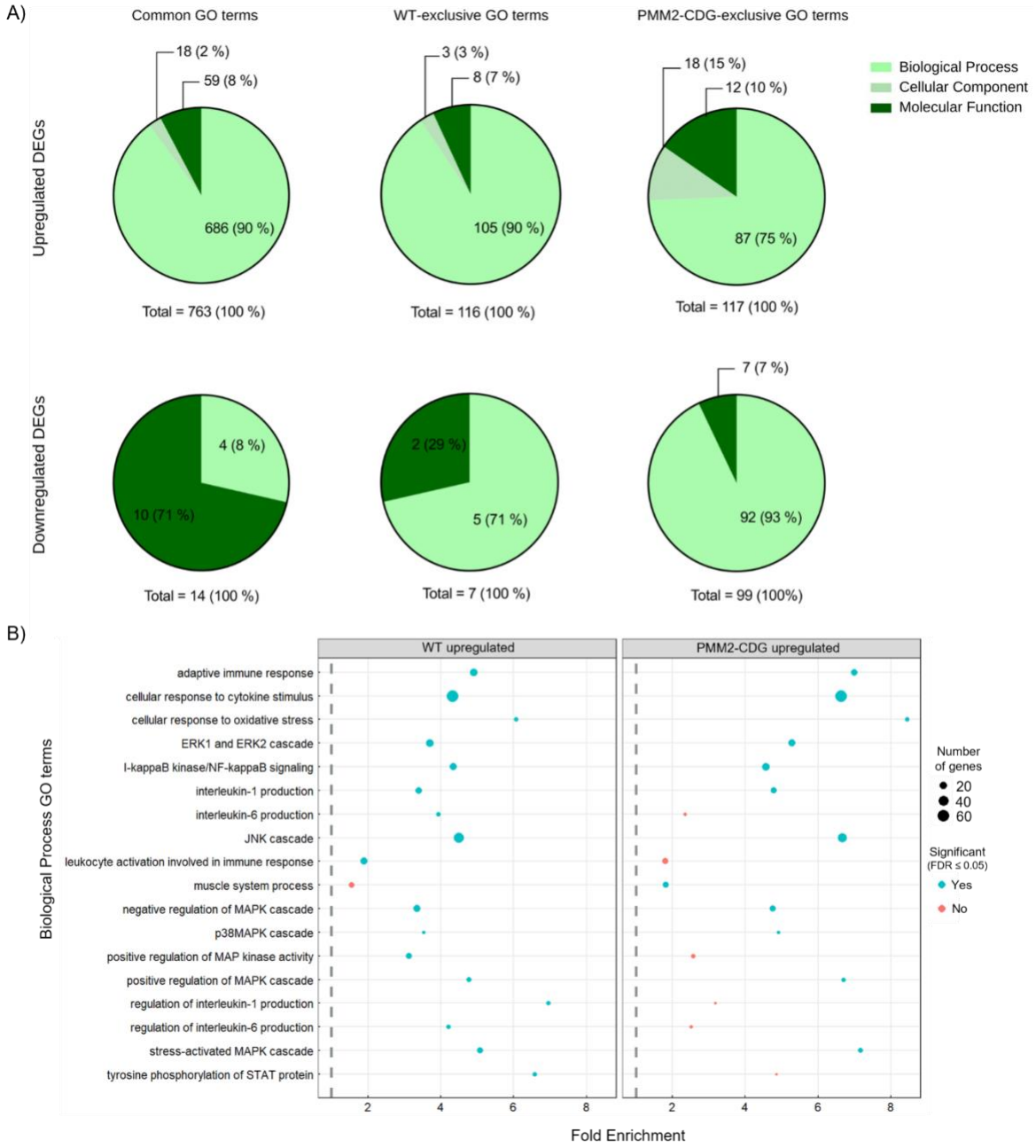

Figure 4.5. Functional enrichment analysis of WT and PMM2-CDG fibroblasts upon TNF-α stimulation. A) Distribution of the gene ontology (GO) terms across the GO domains for the Common enriched functions between WT and PMM2-CDG upregulated terms, WT-exclusive GO terms from the upregulated and downregulated DEG and PMM2-CDG-exclusive GO term from upregulated and downregulated DEG. Light green represents the Biological Process domain; pastel green depicts the Cellular Component domain; and dark green illustrates the Molecular Function domain. Graphical representations were made using GraphPad Prism (version 8, San Diego, USA). B) Fold enrichment plot from the upregulated WT and PMM2-CDG sample groups of the GO terms within the Biological Process domain. Blue dots represent the GO terms which are statistically significant, while the red dots comprise the GO terms which are not statistically significant. The size of the dots comprehends the number of DEG which are inserted in the pathway of the GO term.

Regarding specific enriched upregulated GO terms, it can be observed that immune-related GO terms were enriched in both sample groups (Figure 4.5B, Supplementary File 4.3). All displayed GO terms that are significant in both WT and PMM2-CDG conditions (common GO terms) have a higher fold enrichment value in PMM2-CDG samples than in WT samples, where the difference between the values of the two groups ranges between 1.38 to 3.1 (Figure 4.5B). Particularly, direct or indirect immune-related terms, like regulation of the MAPK cascade, JNK cascade, ERK1 and ERK2 cascade, p38 MAPK cascade, cellular response to oxidative stress, cytokine production and cellular response to cytokine stimulus are some of them. Other particularly important enriched terms have even more pronounced differences but were not included in the graph for better visualization. For instance, the cytokine production term has a fold enrichment of 32.3 in WT samples as opposed to 45.11 for the PMM2-CDG group.

The WT-exclusive GO terms DEG include some interesting upregulated immune-related functions, particularly the positive regulation of interleukin-6 production, leukocyte activation involved in immune response, and interleukin-6 production. Their fold enrichment values are always greater than those of the PMM2-CDG sample groups, in which the adjusted p-values are not statistically significant (Figure 4.5B). In turn, the terms derived from downregulated DEG are limited with some redundancy for morphogenesis and neuro-related terms (Supplementary File 4.4).

Lastly, the PMM2-CDG-exclusive GO terms include terms that can be categorized as key roles in the regulation of proteins that are responsible for the activation of distinct pathways in response to TNF-α stimulation, including positive regulation of JUN kinase activity, serine phosphorylation of STAT protein, and positive regulation of the p38 MAPK cascade. In contrast, positive regulation of transcription, DNA-templated and negative regulation of transcription, DNA-templated are enriched terms from the biological process domain in this downregulated DEG analysis (Supplementary File 4.5). Interestingly, some PMM2-CDG-exclusive terms are related with the muscular system, namely muscle system process (Figure 4.5B). By analysing the log2FC distribution of the overall gene expression in response to TNF-α stimulation in the WT and the PMM2-CDG groups, we can note that the DEG associated with muscular-related terms are, in fact, DEG with extremely different expression levels in response to the stimulus between the two groups and fall into the PMM2-CDG- or WT-exclusive groups. (Supplementary Figure 4.2, Supplementary Table 4.1). A more detailed investigation into the function of these genes evidences roles in muscle development, structure, contraction and regeneration. This observation points out that dysregulated inflammation processes might be the cause or contribute to the clinical manifestations associated to other affected organs or systems.

4.3.5. PMM2-CDG fibroblasts show decreased cytokine expression which might influence predicted interactions with immune cells

We next interrogated if the possible cell-cell interactions between TNF-α stimulated fibroblasts and immune cells could be affected in PMM2-CDG fibroblasts, based on the identified DEG. Thirty-seven DEG that codify receptors and ligands in the fibroblasts were identified. Figure 4.6A shows their possible crosstalk with their pairs present in immune cells based on annotated literature, associated with their log2FC upon stimulation in both WT and PMM2-CDG conditions. We next verified the protein expression of some receptors and ligands to validate the possible cell-cell interactions. Specifically, we found that IL-6, CCL5 and CXCL1 production is activated, and their protein expression significantly increase upon TNF-α stimulation in WT samples (p = 0.004, p = 0.002 and p = 0.024, respectively; Figure 4.6B). However, in the PMM2-CDG, the increase in protein expression of the same cytokines does not augment significantly. This leads to significant and marginally significant lower levels of produced IL-6 (p = 0.03) and CCL5 (p = 0.07), respectively, when compared to WT samples, which can potentially affect their effector functions in neighbour or remote cells. Specifically, low levels of IL-6 might mean that its putative interaction with monocytes, B cells, T cells, dendritic cells, eosinophils, macrophages, NK cells and neutrophils) is impaired while low levels of CCL5 can compromise possible interactions with all abovementioned immune cells, except B cells and CD8+ T cells (Figure 4.6A). Other accessed receptors and ligands codified by DEG either showed no protein expression (IL1B and IL15) or did not show significant differences between WT and PMM2-CDG samples (CD44 and TNFSF10B, data not shown). The assessment of the remaining receptor or ligands identified might highlight other potential impaired interactions between fibroblasts and immune cells.

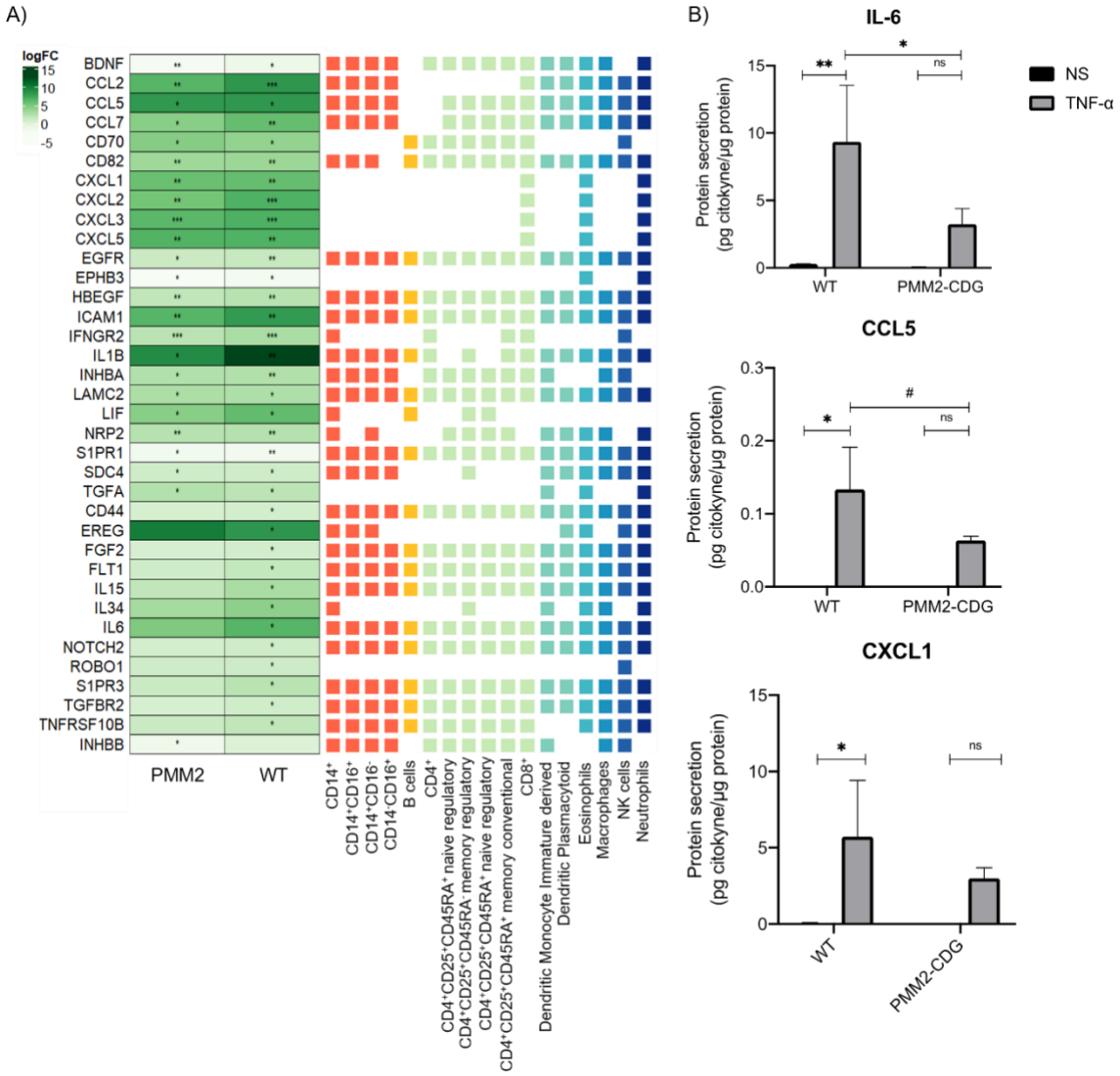

Figure 4.6. Predicted interactions between fibroblasts and immune cells based on the identified DEG and quantification of secreted ligands. (A) Log2 Fold-Change (FC) of differently expressed receptors and ligands upon TNF-α stimulus in PMM2-CDG and WT skin fibroblasts, annotated with a prediction of cell–cell interactions with immune cells. These Log2FC are statistically significant, with FDR < 0.05 (*), FDR < 0.01 (**) or FDR < 0.001 (***). The receptor and ligands represented include common DEG between PMM2-CDG and WT samples (significant in both conditions), WT-exclusive (only significant in WT) and PMM2-CDG DEG (only significant in PMM2) upon TNF-alpha stimulation. (B) Cytokine production of NS and TNF-α stimulated samples (t = 24 h) measured by ELISA (IL-6) or by a Mix and Match LegendPlex kit (CCL5 and CXCL1). Data are represented as the mean ± SD (n = 3). Statistically significant values were accessed by a one-way ANOVA with Dunnett's multiple comparisons ($p < 0.05$ (*), $p < 0.01$ (**), $0.05 < p \leq 0.1$ (#). Normalized values were obtained by dividing the concentration of cytokines (pg/ml) by the concentration of total protein (mg/ml).

4.3.6. TNF-α signalling is possibly deregulated in PMM2-CDG fibroblasts through the p38 MAPK, ERK and JNK pathways

The functional enrichment analysis and evaluation of stimulation products points towards errors in TNF signalling pathways in PMM2-CDG. To study the potential deregulated intracellular mechanisms, we first investigated the gene expression of the signalling, adaptor and regulatory proteins involved in the downstream pathways of the TNFR1 (Figure 4.7A). Gene expression results indicated that, upon TNF-α stimulation, *TRAF5*, *TAB2* and *FOSL2* expression increases in WT but not PMM2-CDG samples. In contrast, *TRAF2*, *RELA* and *FOS* increase their expression in PMM2-CDG but not WT samples. Even though significantly increased in both groups, other genes codifying TNFR1-related signalling proteins also show some slight expression differences. These results indicate that the signalling mediated by the TNFR1-TRADD-TRAF-TAB2 is possibly deregulated. Consequently, we inferred that downstream signalling can also be affected, namely the IKK complex, JNK, p38 and ERK1/2 pathways. To test this, the protein expression of ERK1/2, p38 and -JNK were analysed by western blot. We could note that ERK1/2 and p38 levels diminish upon TNF-α stimulation in WT samples (p = 0.006 and p = 0.02, respectively) but the same does not happen in the PMM2-CDG. Additionally, the baseline level of P38 protein and ERK1/2 is significantly higher in the case of the former (p = 0.003), and tends to be higher, in the case of the latter, in WT compared to PMM2-CDG fibroblasts (Figure 4.7B). JNK2 levels were assessed in one patient and found to be decreased before and after stimulation (Supplementary Figure 4.3). On the contrary, we could not detect differences in IKBα expression (data not shown) nor analyse the NF-κB subunits.

The observed differences are in line with the previously noted impairment in proinflammatory cytokine and chemokine production since both JNK and ERK1/2 (through the AP1 complex composed of Fos and Jun dimers) as well as p38 are important for transcription and regulation of pro-inflammatory genes [43]–[45]. Importantly, our transcriptomic results provide no evidence that TNF-α-induced cell death programmes are activated in neither WT nor PMM2-CDG samples, suggesting that their activation might be independent of the TNFR1 glycosylation status. Figure 4.7C provides an illustration of how TNFR1 intracellular signalling might be affected in PMM2-CDG based on our gene and protein expression.

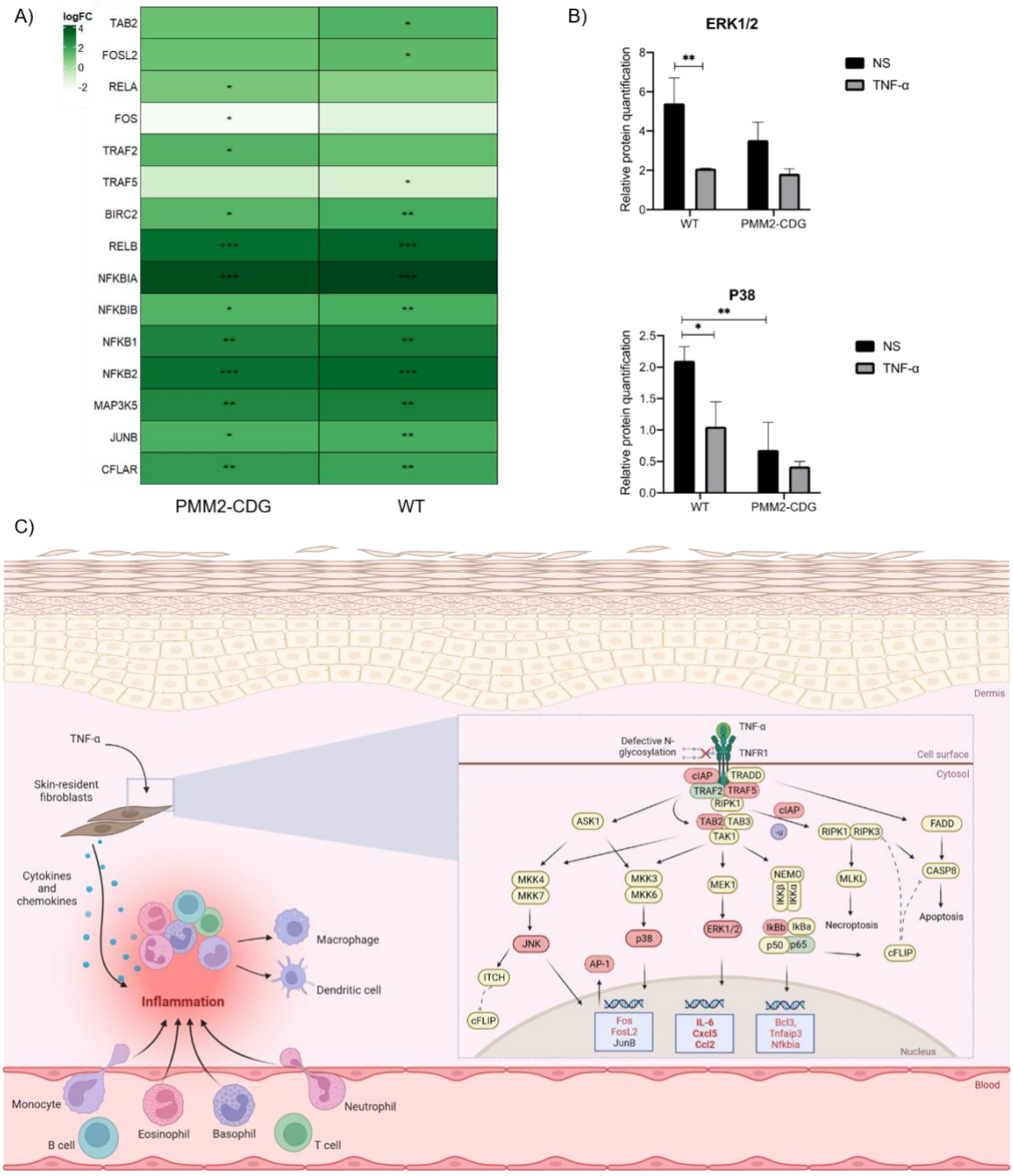

Figure 4.7. Mechanism of deregulated TNFR1 signalling in PMM2-CDG. (A) Heatmap of the log2FC of signalling, adaptor and regulatory proteins involved in TNFR1 signalling. (B) Relative protein quantification of cell signalling proteins (namely p38, and extracellular signal-regulated kinase (ERK) 1/2 of NS and TNF-α stimulated fibroblasts (t = 30 min) samples by western blot. The detection of α-tubulin was for band normalization (housekeeping protein normalization method). Statistically significant values were accessed by a one-way ANOVA with Dunnett's multiple comparisons ($p < 0.05$ (*), $p < 0.01$ (**)). (C) Illustration of the deregulated signalling, adaptor and regulatory proteins involved in TNFR1 signalling. Downregulated expression is shown in red, upregulated expression is shown in green. The proteins whose expression does not differ between WT and PMM2-CDG or where is no indication of their expression are highlighted in yellow. For the proteins where differences were identified or inferred (down or upregulated in PMM2-

CDG), when the levels of expression are indicated by only gene expression levels, these are outlines by a dotted border; when the expression levels were identified by protein expression, these are outlined by a continuous border. Expression levels confirmed by both gene and protein levels are in bold.

4.4. Discussion

One of the pitfalls of PMM2-CDG clinical care and research is the profusion of wide-ranging clinical manifestations and their heterogeneity for which the underlying pathology is still not fully understood. This is the case for the occurrence of recurrent, severe, sometimes life-threatening infections and other immunological alterations in the earlier stages of patients' lives. In this study, we proposed to investigate if and how the defective glycosylation that characterizes PMM2-CDG impacts immune molecular mechanisms and function resorting to patient-derived skin fibroblasts and focusing on the TNF-α-mediated signalling.

Both acute and chronic inflammation are known to be linked to glycosylation changes. On the first case, the glycosylation status of certain acute-phase proteins can dictate their immunomodulatory activity [46]. On the latter case, a bi-directional influence exists since, on the one side, a constant inflammatory environment modulates the local carbohydrate repertoire, and, on the other side, these glycan alterations will, in turn, impact the normal functioning of the immune system [47]. Hinting for the potential dysregulation of the inflammatory response in PMM2-CDG, the N-glycosylation profile of our patient cohort was significantly deficient upon the inflammatory stimulus. Additionally, even though the expression of TNFR1 decreases after TNF-α stimulation equally in control and PMM2-CDG samples, as observed by others [48], we detected structural differences in this receptor between the two groups. We detected the presence of a TNFR1 fragment in stimulated PMM2-CDG fibroblasts contrasting with the control sample. TNF-α converting enzyme (TACE) is a membrane bound protein responsible for cleaving TNF-α and its receptors releasing soluble forms. In the case of soluble TNFR1 (34 kDa), it has unfavourable effects increasing inflammation [49]. Additionally, the glycosylation of a recombinant form of TACE has been shown to modulate its activity, with less glycosylated enzyme showing more catalytic activity [50]. Even though this finding remains to be verified in its membrane-bound form, a potential altered TACE glycosylation can help explain the presence of a fragmented TNFR1 form in PMM2-CDG samples.

Apart from the structural differences observed between PMM2-CDG and control fibroblasts under TNF-α stimulation, transcriptional differences were also observed. Specifically, from our cohort of three patients, two of them have a pronounced transcriptional difference to the WT samples, while the transcriptional profile of another patient (GM20945) is more aligned with the controls, under both non-stimulated and stimulated conditions. This observation is well explained

when considering the patients' genotype. In fact, the two samples that cluster together have a more common and severe heterozygous mutation (R141H) [51], whereas the genotype of the "outlier" PMM2-CDG sample is associated with milder cases of the condition with fewer glycosylation defects and some degree of walking and autonomous abilities [52]. Further supporting this observation is our previous work that resorted to the administration of an e-questionnaire targeting immunological events in CDG (ImmunoCDGQ). The patient sample included 122 PMM2-CDG patients from which 41 (34%) harbouring the R141H variant were identified to have immune-related manifestations [35]. These include mostly frequent or severe infections and allergies as well as some frequently unexplained fever episodes and altered responses to vaccination. Besides, several reported cases bearing this variant have been associated with septic events [53]. Therefore, immunological involvement in PMM2-CDG seems to be dependent on not only the patient genotype but also on disease severity.

The analysis of DEG following the TNF-α stimulus contributed to a better understanding of the immunological (dys)functioning of PMM2-CDG samples in response to a pro-inflammatory environment. Even though both WT and PMM2-CDG fibroblasts respond to the TNF-α stimulus, there are three striking differences in this response: 1) not only there is an immediate difference in the number of TNF-α-responsive genes between the WT and PMM2-CDG groups, 2) but also there are genes that are exclusively up or downregulated in one or the other group. Additionally, 3) genes that respond in both WT and PMM2-CDG groups do not necessarily have similar magnitudes of expression changes. These factors indicate gene regulation defects upon the inflammatory stimulus.

Proceeding with further analysis, our results show that the differences in gene expression translate into disparities at the functional level. The enrichment analysis shows that TNF-α activated standard pathways according to the literature, including NF-κB signalling and several MAPK cascades (p38, JNK, and ERK1 and ERK2 cascades) in both healthy and PMM2-CDG samples [54]–[57]. Nonetheless, the differences between the fold enrichment values for these terms are quite pronounced. This suggests these interconnected pathways are dysregulated in PMM2-CDG individuals. These pathways are intimately associated with the activation and regulation of various proinflammatory cytokines [44], [58], [59], which is in agreement with the enrichment of several cytokine-related processes. Accordingly, we found that PMM2-CDG fibroblasts produce lower levels of IL-6 and CCL5 after TNF-α stimulation. Deficiencies in these molecules can widely hinder the immune response, given their importance in host defence and their immunomodulatory roles exerted in immune cells. IL-6 is a cytokine with pleiotropic immune functions with important roles in inflammation and against several types of infections [60], [61]. CCL5, also known as RANTES, is a chemokine that recruits leukocytes to sites of inflammation [62]. Concomitantly, the term 'leukocyte activation involved in immune response' is only enriched in WT samples. This observation is supported by studies in a N-glycosylation deficient mouse model which showed

lower neutrophil extravasation under an inflammatory response when compared with control mice [63]. Hence, the decreased levels of these molecules and, sequentially, their potential hampered leukocyte activation, might partially explain the previously reported recurrent and severe infections of PMM2-CDG patients [35]. Nevertheless, the analysis of other proteins codified by the identified DEG, especially those with different expression between PMM2-CDG and control samples might allow to identify other compromised immune players and potentially affected interactions with immune cells.

To corroborate the above-mentioned observations, we further explored the expression of TNFR1 signalling players. Besides highlighting several significant differences between the response to TNF-α of several genes that codify proteins downstream to TNFR1 (like TRAF2, TRAF5 and TAB2) we also show that PMM2-CDG fibroblasts express less of ERK1/2, p-38 and JNK signalling proteins. This is not only concordant with the lower expression of cytokines previously mentioned given their functions [44], [45], [56], but also with the impaired expression of other genes and transcription factors that are activated by these signalling pathways. In fact, we detected that FOS and FOSL2, which codify the Fos protein, are significantly downregulated in PMM2-CDG which are regulated by ERK1/2 [64]–[66]. Similarly, Jun proteins are probably less or not activated given the downregulation of JNK [67]. Therefore, we anticipate that they will have lower levels of the AP-1 complex composed of Fos and Jun proteins [68]. Importantly, the AP-1 complex is known to regulate the transcription of cytokines and factor involved in skin homeostasis, regeneration, and inflammation [68], [69]. Even though we were not able to investigate the protein expression of NF-κB, our results show that there are several related genes with a deregulated expression. This is line with previous observation in microglia showing that potential TNFR1 glycosylation deficiencies affect its NF-κB transcription factor's activity [70]. Further studies are needed to clarify the complete signalling dysregulation of all the analysed pathways, including expression and functionality of the transcription factors.

Our comprehensive methodology included big-data analysis of the transcriptome, allowing the analysis of the overall behaviour of the cell under inflammatory conditions. Besides the expected TNF-α-mediated activation of several immune-related signalling pathways, strikingly, processes related to the muscle system were enriched. The genes related with these processes have roles in muscle development, structure, contraction, and regeneration. Both WT and PMM2-CDG fibroblasts overexpress TGF-α and EGFR upon TNF-α stimulation known to mediate the inflammatory events of wound healing and regeneration [71]. During these processes, fibroblasts are activated to proliferate and differentiate in myofibroblasts have smooth muscle-like features [72]. In a balanced and well-regulated response, myofibroblasts dedifferentiate into fibroblasts though several processes, including by anti-fibrotic effects of FGF-2 [73]. Interestingly, FGF-2 is overexpressed in WT but not PMM2-CDG samples indicating a possible deregulation that might lead to the accumulation of myofibroblasts. In fact, the unbalanced expression of the described

players has been associated with tissue fibrosis [74], [75]. Therefore, this work also highlighted possible deregulated mechanisms that could be behind other signs and symptoms related with other organs or system, like liver fibrosis that affects some PMM2-CDG patients [76].

This study has some limitations. Our small patient sample size might not be representative of the true dysfunction usually found in PMM2-CDG patients due to its small size. Indeed, further analysis with more representative groups per genotype, phenotypes and severities would be warranted to understand if there is a correlation with immunological dysregulation. Furthermore, the selected sequencing method has a reduced number of target reads as the final product and assesses genes with 3' end alternative splicing which impairs the capacity to detect transcripts with lower expression levels. A more powerful sequencing protocol would allow for a more comprehensive view of the PMM2-CDG immune dysregulation. Nevertheless, as far as we are aware, this is first exploratory transcriptomics study aimed at elucidating the underlying impaired mechanisms in the immunological response of PMM2-CDG patients and allowed us to identify potential deficient pathways in response to a proinflammatory stimulus.

This study proves the concept that the glycosylation deficiencies associated with PMM2-CDG can affect downstream signalling of cell surface receptors, in this case TNFR1 signalling. Nevertheless, the exact mechanism underlying signalling dysregulation are still elusive. Two hypotheses can be advanced. As shown by our results, alteration of cell surface or receptor glycosylation might influence the structure of the receptor affecting its downstream events. Alternatively, it can impact ligand binding affinity as shown in other contexts [70], [77]. Studies targeted at clarifying this matter are warranted. Besides, treatment with epalrestat – an aldose reductase inhibitor – of patients' fibroblasts bearing the R141H/E139K (interestingly, in this case, the same cell line used in our study) and R141H/N216I genotypes, increased their PMM2 activity [78]. While this treatment shows to have clinical benefit at the neurological and biochemically level in patients, it would be noteworthy to understand if it can solve the immune (dys)function in PMM2-CDG patient. Besides this work highlights intracellular signalling proteins as potential targets for immunotherapy for PMM2-CDG.

4.5. Conclusion

This work provides new insights into the glycan-related immunopathological mechanisms of PMM2-CDG patients. Using a transcriptomics approach and a series of experimental validation, it identified key defects in distinct pathways, namely, p38, ERK1/2 and JNK2, following TNF-α stimulus which translate into functional defects, namely the secretion of immunomodulatory cytokines. Additionally, possible mechanistic implications and research avenues for other clinical

manifestations related to other systems have been uncovered. Importantly, this study identifies potential targets for immunotherapy for PMM2-CDG and provides an experimental design to study the immunopathology in other immune-related CDG.

4.6. References

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CHAPTER 5.

PATIENT REPORTED OUTCOMES FOR PHOSPHOMANNOMUTASE 2 CONGENITAL DISORDER OF GLYCOSYLATION (PMM2-CDG): LISTENING TO WHAT MATTERS FOR THE PATIENTS AND HEALTH PROFESSIONALS

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Abstract

Background: Congenital Disorders of Glycosylation (CDG) are a growing group of rare genetic disorders. The most common CDG is phosphomannomutase 2 (PMM2)-CDG which often has a severe clinical presentation and life-limiting consequences. There are no approved therapies for this condition. Also, there are no validated disease-specific quality of life (QoL) scales to assess the heterogeneous clinical burden of PMM2-CDG which presents a challenge for the assessment of the disease severity and the impact of a certain treatment on the course of the disease.

Aim and methods: This study aimed to identify the most impactful clinical signs and symptoms of PMM2-CDG, and specific patient and observer reported outcome measures (PROMs and ObsROMs, respectively) that can adequately measure such impact on patients' QoL. The most burdensome signs and symptoms were identified through input from the CDG community using a survey targeting PMM2-CDG families and experts, followed by family interviews to understand the real burden of these symptoms in daily life. The list of signs and symptoms was then verified and refined by patient representatives and medical experts in the field. Finally, a literature search for PROMs and ObsROMs used in other rare or common diseases with similar signs and symptoms to those of PMM2-CDG was performed. Those scales could be applied directly to the PMM2-CDG population or adapted to create the first PMM2-CDG-specific QoL questionnaire.

Results: Twenty-four signs/symptoms were identified as the most impactful throughout PMM2-CDG patients' lifetime. We found 239 articles that included tools to measure those community-selected PMM2-CDG symptoms. Among them, we identified 80 QoL scales that address those signs and symptoms and, subsequently, their psychometric quality was analysed.

Conclusion: Identifying the impactful clinical manifestations of PMM2-CDG, along with the collection of PROMs/ObsROMs assessing QoL using a creative and community-centric methodology are the first step towards the development of a new, tailored, and specific PMM2-CDG QoL questionnaire. These findings can be used to fill a gap in PMM2-CDG clinical development. Importantly, this methodology is transferable to other CDG and rare diseases with multiple signs and symptoms.

Keywords: Outcome assessment, Patient reported outcomes, Observer reported outcomes, Quality of life, Rare diseases, PMM2-CDG, People-centricity

5.1. Introduction

The World Health Organization defines Quality of Life (QoL) as "an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" [1]. One of the aspects of QoL is health-related quality of life (HrQoL). HrQoL is a multi-domain concept that encompasses physical, emotional, mental, and social functioning. It can be measured in a variety of ways, such as general scales, disease- or symptom-specific tools, which reflect upon the subjective perspective of a person regarding their condition [2]. Although general scales can be used for different diseases, they are less sensitive to detect small, yet important clinical differences in treatment effects [3]. These important differences are better measured using disease- or symptom-specific HrQoL scales, which will be more sensitive as they assess specific hallmarks of the disease or symptom. Concerning rare diseases, the study of QoL is challenging due to methodological issues as well as to limited literature on those conditions. Small patient populations, disease heterogeneity and scarcity of medical knowledge and specialists hamper the understanding of the burden of these diseases [4], [5]. This highlights the importance of ensuring a community-centric approach, including the professionals' experience and the patients' voice. Involving both stakeholder groups not only maximizes data collection but also data meaningfulness, ultimately contributing to the creation of sensitive and disease-tailored QoL tools. This is vital to delivering and appraising potential therapeutics.

Patient-reported outcome measures (PROMs) and observer-reported outcome measures (ObsROMs) are quantitative tools to obtain reports of patient outcomes directly from patients or their family/professional caregivers, respectively. They have been increasingly utilised as clinical endpoints, particularly with the aim to detect changes in the HrQoL in response to treatments [6]. They allow a deeper understanding of treatment impact and report domains that are not just clinically important but also meaningful for the patients [7]. They have been extremely useful, especially in chronic illnesses [6], [8] and are recommended by regulatory agencies such as the Food and Drug Administration and the European Medicine Agency, to support the approval of new therapies and medical labelling claims [9], [10].

Congenital disorders of glycosylation (CDG) are a growing family of rare diseases that affect the synthesis and attachment of sugar 'trees' (glycans) of proteins and lipids. These defects often have severe, multi-organ implications for the patients, since about 50% of human proteins are glycosylated and glycans play essential roles in all biological processes [11]. PMM2-CDG is the most common CDG, and it is due to autosomal recessive variants in the PMM2 gene, which encodes the enzyme phosphomannomutase 2, essential for N-glycosylation. This enzyme is responsible for the synthesis of N-linked oligosaccharides by converting mannose 6-phosphate

to mannose 1-phosphate [12]. PMM2-CDG clinical presentation is dominated by neurologic abnormalities such as psychomotor disability, seizures, hypotonia and ataxia, besides multiple organ involvement resulting in chronic disability, poor QoL and premature death [13]. Some potential treatments, such as liposome-encapsulated mannose 1-phosphate administration, are undergoing clinical studies [14]. More recently, a trial with acetazolamide showed improvement of the ataxia [15]. Moreover, in a single-patient paediatric trial with epalrestat, improvements in ataxia and also in growth were observed [16]. However, specific tools are needed to measure QoL in PMM2-CDG to understand if a treatment has a significant impact.

Currently, there are no disease-specific QoL PROMs/ObsROMs for PMM2-CDG. Here, we used a community-centric approach, involving CDG medical professionals and families in the design and conduction of the study. We aimed to gather PROMs and ObsROMs that are specific for the most impactful PMM2-CDG clinical signs and symptoms. For that purpose, we surveyed PMM2-CDG families and clinicians following PMM2-CDG patients to understand which are the most onerous signs and symptoms, and interviewed families to understand the real burden of those clinical manifestations in everyday life. Considering the input of these stakeholders, we reviewed the literature about the PROMs and ObsROMs used in other rare and common diseases with similar signs and symptoms to PMM2-CDG. Those tools could potentially be validated and applied directly to the PMM2-CDG population or adapted to create the first PMM2-CDG-specific QoL questionnaire.

5.2. Materials and Methods

5.2.1. Set up of the patient and medical advisory committees

Two advisory committees were established to provide expert insights regarding the understanding and particularities of the disease and to guide decision making throughout this project. Patient experts, specifically 11 family caregivers, and 9 medical experts were invited to participate in the committees. A summary of the project and an explanation of their roles were provided if they agreed to participate. Communications were mainly done by email or by video calls when necessary.

5.2.2. Quantitative analysis of PMM2-CDG symptoms' impact (PMM2-CDG Symptoms' Impact Survey)

A survey was constructed to assess the impact of the signs and symptoms from infancy to adulthood. Two versions were used, one targeting PMM2-CDG families and the other targeting

medical experts. Electronic samples of the survey are available at https://www.surveymonkey.com/r/HCPCOM (medical experts' version) and https://www.surveymonkey.com/r/PATCOMM (version adapted to families). The survey included an exhaustive list of signs and symptoms reported in the OMIM database (MIM: 212065) but also reported by CDG families. Family experiences included both personal communications and social media reports in the CDG Global Alliance Facebook Group, a social media platform uniting worldwide CDG patients and professionals perceived as a safe environment where the community openly shares questions, concerns, and experiences. The information derived from this group complies with the terms and conditions of the platform and with the privacy settings of the participants. It was shared in a voluntary manner with all participants of the group and fell under the objectives of the group (i.e., promoting shared knowledge between families, doctors, and researchers). This was a complementary step to validate and complete the information collected through other sources, therefore, the information was not transcribed and thus is not traceable and constitute no risk of harm to the participants. Anonymity was maintained in all instances. Printed surveys were distributed at the beginning of the 4th World Conference on CDG for Families and Professionals, held in Lisbon on the 26th and 27th July 2019. Given that most PMM2-CDG patients are unable to provide self-reports due to the fact that 1) most are of paediatric age and 2) have considerable cognitive impairment, patients' views were evaluated and conveyed by patients' families. Observer and proxy reports have been commonly used in studies where self-reports cannot be obtained [17–19]. Therefore, patients' caregivers answered the survey voluntarily following written and verbal information about the study. Respondents were asked to classify the daily life impact of each of the symptoms/clinical manifestations on a scale of 1 - "No impact" to 5 - "Extremely negative impact" considering each phase of the patient's life (infancy: 0-3 years; childhood: 4-10 years; adolescence: 11-17 years; and adulthood: 18 years and older). To increase data collection, respondents could answer to more than one age range as long as they felt comfortable and confident in doing so (e.g., the caregiver of an adolescent patient could answer both the infancy, childhood and adolescent sections). An "I don't know/cannot answer" option was available to improve data collection and quality. Additionally, respondents were given the chance to share relevant information that they felt was missing in the survey by including an optional text field: "If there are other symptoms you find impactful, please list them here and rate the magnitude of their impact (using the same scale)". The surveys were collected by the end of the conference. The final impact level of each clinical manifestation was calculated using the mean value of all respondents for each given age range. The 7 symptoms with higher impact level for each age range for both families and professionals were summed up, yielding a final list of 16 unique impactful symptoms (7 symptoms x 4 age ranges x 2 target groups = 56 – 40 duplicates = 16 unique symptoms). To analyse the differences between the families' and clinicians' perspectives, for each sign/symptom, a two-way ANOVA test with multiple comparisons and

Sidak's correction was performed yielding an adjusted p-value. Statistical significance was considered if adjusted p-value < 0.05.

5.2.3. Qualitative insights of PMM2-CDG symptoms' impact

Interviews were designed and led to gather insights about the real-word impact of the signs and symptoms identified in the survey as being "the most impactful" from families' perspectives (Supplementary Table 5.1). All medical and difficult terms were referred to in lay-language and further explained when required by the participant to ensure their understanding. Deidentified transcripts were obtained from seven interviews of mothers of PMM2-CDG patients which were part of our patient committee. Demographics of the patients included in the interviews are available in Supplementary Table 5.2. The interviewees were prompted to share patient experiences in greater depth. Hence, questions were open-ended to avoid bias and were not read verbatim to permit free-flowing discussion. The collected insights were used to guide our article selection to make it more specific and targeted to the patient's needs.

5.2.4. Review of the literature

5.2.4.1. Search strategy

The community-identified burdensome signs and symptoms guided a literature review strategy to identify and gather specific PROMs and ObsROMs. The PubMed database was queried with pre-defined search terms on September 11th, 2020. The search query was based on three groups of search terms: 1) QoL related, 2) PROMs/ObsROMs related terms, and 3) impactful signs and symptoms previously identified - connected by the Boolean operator "AND" (Supplementary Table 5.3). Keywords within the same group were connected using the operator "OR". For some signs and symptoms and given the fact that PMM2-CDG is a rare metabolic disorder, the keywords "metabolic" or "rare disease" were added to the combination to provide more specific results. Resulting articles from the search were exported and duplicated articles were eliminated. References of relevant articles were screened, and additional articles were included by author referral (Figure 5.1).

5.2.4.2. Study selection and data extraction

Resulting articles were screened based on predefined inclusion and exclusion criteria. Studies had to be written in English and measure HrQoL for one or more of the previously identified impactful signs/symptoms, by means of a PROM or ObsROM. Articles using clinician-reported outcomes, performance outcomes, interviews and reviews were excluded. Furthermore, studies reporting caregiver QoL and that explicitly affirmed the use of non-English translations of

the PROMs/ObsROMs were excluded. Nevertheless, articles describing the use of foreign (non-English) questionnaires for whom an English translation is available were included (e.g., Deglutition Handicap Index, Izumo scale). Article titles and abstracts were screened and, subsequently, the full-text versions of the remaining articles were evaluated according to the inclusion/exclusion criteria (Figure 5.1). Article content analysis and data extraction was performed by a group of 4 researchers, specifically regarding the PROMs employed to measure the QoL, the participants' cohort and disease(s)/sign(s)/symptom(s) assessed. For some sign(s)/symptom(s), no specific tool was found. In these cases, some adequate items or subscales were secondarily captured by the inclusion of other tools.

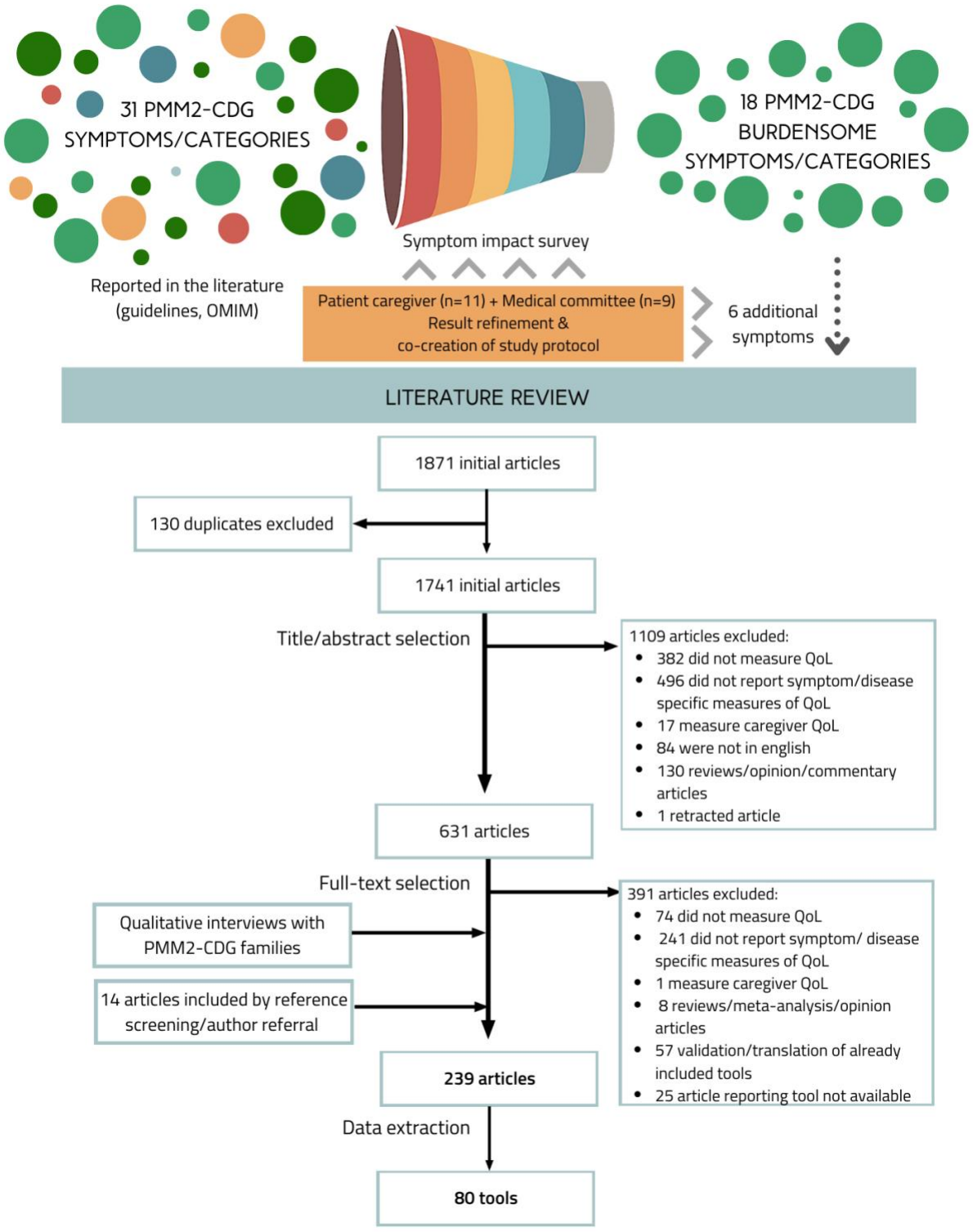

Figure 5.1. Overview of the study workflow.

5.2.4.3. Quality analysis

The purpose of the quality analysis was not to perform a systematic review of the psychometric properties of the included instruments, but rather to identify and compare them in terms of their psychometric properties, namely Content, Criterion and Construct Validity, Internal Consistency, Agreement, Reliability, Responsiveness, Floor and Ceiling effect and Interpretability (Supplementary File 5.1). To do so, this analysis was based on the original development and/or validation articles of the instruments. Thus, translations or validations to other languages besides English were not considered. One instrument could not be evaluated (Scoliosis Research Society-30) as its development and validation articles were not available. Also, three instruments (Short Inflammatory Bowel Disease Questionnaire, Pittsburgh Insomnia Rating Scale, and College of Optometrists in Vision Development Quality of Life Questionnaire) were evaluated exclusively based on the available abstracts.

The analysis was made using the Quality Criteria for Measurement Properties of Health Status Questionnaire developed by Terwee et al., (2007) for the design, methods, and outcomes of the development and validation studies [20]. Based on these criteria, each psychometric property was evaluated with (+) - positive rate; (?) - indeterminate or doubtful rate; (-) - negative rate; or (0) - no information available. Some adaptations of the criteria were needed:

1) for Construct Validity evaluation, the criteria for a positive rating requires that specific hypotheses have been formulated and at least 75 % of the results are in accordance with them. However, given that for the majority of the articles, hypotheses were not explicitly presented by the authors, we had to analyse if the goal of development and/or validation of the instrument was met;

2) for Internal Consistency, the criteria of the sample size being N = 7 × the number of items and N >100 was not considered for two reasons. First, because of the great variability in the number of items between questionnaires, and second, because we are dealing with symptom/condition-specific questionnaires, and therefore, the clinical samples of validation articles are usually smaller than if we were dealing with a healthy population.;

3) when in doubt about meeting less objective criteria (e.g., convincing arguments that gold standard is ''gold'', for Criterion Validity), the properties were classified with a positive rating if the validation methodology was clearly described and the authors clearly justified their conclusions well.

5.3. Results

5.3.1. Selection of the most impactful symptoms by the community and the expert committees

The PMM2-CDG symptoms' impact survey had 42 respondents: 23 family representatives and 19 PMM2-CDG medical experts. A list of the topmost impactful symptoms was then obtained considering the sum of the 7 most impactful manifestations across the 4 age ranges considered according to both families and clinicians and excluding duplicates. This resulted in a list of 16 signs and symptoms (Table 5.1).

There was a good level of agreement between the perspectives of families and clinicians, particularly for the infancy period. For this age group, only seizures were rated with a statistically significant difference ($q < 0{,}001$) between families (IS = 1.78, n = 18) and clinicians (IS = 3.67, n=18). Significant differences between the views of both stakeholders were predominant for the childhood group. During this timeframe, dysphagia (IS = 2.00, n=19 for families and IS = 3.93, n = 15 for clinicians, q = 0.003) and seizures (IS = 2.05, n = 19 for families and IS = 3.87, n = 16, q = 0.001) were perceived to have a much higher negative impact by clinicians while for families a moderate negative impact was reported. The presence of sex development issues was also associated with a bigger impact by the medical doctors (IS = 2.53, n = 15) in comparison to PMM2-CDG families (IS = 1.14, n = 14; q = 0.031). Concerning stroke-like episodes, an extremely negative impact on adolescent PMM2-CDG patients was perceived by clinicians (IS = 4.08, n = 12) while none to slight negative impact (IS = 1.25, n = 4) were alleged by family members (q = 0.013). The same tendency was seen for the adult group. Lastly, although not statistically significant, clinicians tended to rate kyphosis/scoliosis with a more pronounced negative impact than families during adolescence and childhood. The same happened regarding peripheral neuropathy, particularly in the childhood and adulthood group (Table 5.1).

The analysis of the qualitative data shared on the survey as well as the revision of the most impactful signs and symptoms by the family and medical committees resulted in the inclusion of 6 additional clinical manifestations, namely sleep disturbances, liver problems, coagulopathy, food allergies, cardiomyopathy, and pericardial effusion.

Table 5.1. Impact scores (n) for selected signs and symptoms by age range and according to families and clinicians' perspectives. Legend: 1-<2 – No or slight negative impact; 2-<3 – Moderate negative impact; 3-<4 – Negative impact; 4-<5 – Extremely negative impact. q - adjusted p-value.

		Impact score - weighted mean (n)							
Signs & Symptoms	Group	Infancy	q	Childhood	q	Adolescence	q	Adulthood	q
Hypotonia	Families	4.7 (19)	0.795	4.0 (21)	0.896	3.4 (5)	0.999	4.5 (2)	0.527
	Clinicians	4.3 (19)		3.8 (17)		3.5 (13)		3.3 (15)	
Developmental delay	Families	4.7 (19)	0.970	4.2 (21)	0.979	4.0 (5)	0.826	3.0 (2)	0.891
	Clinicians	4.5 (19)		4.0 (17)		3.4 (14)		3.7 (14)	
Ataxia	Families	4.5 (19)	0.702	4.0 (21)	1.000	3.4 (5)	0.245	4.0 (2)	0.976
	Clinicians	4.1 (19)		4.1 (17)		4.5 (13)		4.5 (13)	
Dysarthria/Speech delay	Families	4.5 (19)	0.547	4.1 (20)	0.992	4.0 (5)	0.999	3.0 (2)	0.685
	Clinicians	3.9 (18)		3.9 (15)		4.1 (13)		4.1 (14)	
Intellectual disability	Families	3.8 (18)	1.000	3.8 (21)	0.720	3.8 (5)	0.979	3.0 (2)	0.733
	Clinicians	3.7 (18)		4.2 (17)		4.1 (14)		4.1 (14)	
Ophthalmological problems	Families	3.7 (18)	0.998	3.2 (21)	0.182	3.6 (5)	0.994	4.5 (2)	0.966
	Clinicians	3.8 (19)		4.1 (17)		3.8 (13)		3.9 (13)	
Infections	Families	3.7 (19)	0.946	2.6 (20)	1.000	2.8 (5)	0.962	2.0 (2)	0.953
	Clinicians	3.4 (18)		2.5 (15)		2.4 (13)		2.6 (14)	
Peripheral neuropathy	Families	2.9 (18)	0.932	2.5 (17)	0.094	3.3 (3)	0.963	2.0 (2)	0.186

	Clinicians	2.5 (17)		3.7 (16)		3.9 (11)		4.3 (13)	
Dysphagia	Families	2.7 (18)	0.178	**2.0 (19)**	**0.003**	2.0 (4)	0.505	1.0 (2)	0.158
	Clinicians	3.8 (17)		**3.9 (15)**		3.3 (13)		3.5 (13)	
Hyperthermia episodes	Families	2.7 (17)	0.985	2.2 (20)	0.997	3.5 (2)	0.630	3.5 (2)	0.667
	Clinicians	2.5 (16)		2.1 (15)		2.2 (12)		2.3 (13)	
Behavioural problems	Families	2.5 (19)	0.546	3.0 (19)	0.769	2.8 (5)	0.400	3.0 (2)	0.962
	Clinicians	3.2 (18)		3.6 (15)		4.1 (13)		3.7 (13)	
Kyphosis/Scoliosis	Families	1.8 (18)	0.609	2.1 (19)	0.121	2.0 (5)	0.053	3.5 (2)	0.965
	Clinicians	2.4 (17)		3.2 (16)		3.9 (14)		4.1 (14)	
Seizures	Families	**1.7 (18)**	**<0.001**	**2.0 (19)**	**0.001**	2.0 (4)	0.137	1.0 (2)	0.109
	Clinicians	**3.7 (18)**		**3.9 (16)**		3.8 (13)		3.5 (13)	
Stroke-like episodes	Families	1.5 (15)	0.134	2.7 (17)	0.250	**1.2 (4)**	**0.013**	1.0 (2)	0.078
	Clinicians	2.8 (17)		3.7 (16)		**4.1 (12)**		3.9 (13)	
Osteopenia	Families	1.5 (15)	0.839	1.9 (12)	0.320	3.3 (3)	0.999	3.5 (2)	0.996
	Clinicians	1.9 (17)		2.7 (16)		3.5 (14)		3.8 (15)	
Sex development disorders	Families	1.2 (13)	0.777	**1.1 (14)**	**0.031**	4.0 (5)	0.913	4.5 (2)	0.878
	Clinicians	1.7 (16)		**2.5 (15)**		3.5 (13)		3.6 (14)	

5.3.2. Families' perspectives about the real-world impact of the most impactful symptoms

Semi-structured interviews with open-ended questions were led with family members of PMM2-CDG patients which allowed them to express the burden of living with the disease and the consequences of specific clinical manifestations in family life. The summary results of the interviews encompassing the experiences with the complete list of clinical manifestations are described in Supplementary File 5.2. This information allowed us to refine and further tailor our article and QoL assessment tools selection to the experiences of PMM2-CDG families. As an example, osteopenia/osteoporosis, clinically characterized by low bone density, occurs in PMM2-CDG patients at a later stage in life, but with significant consequences for the patient's daily life. One family member stated that "[osteoporosis] *causes pain when she is sitting in the wheelchair as well as getting up and sitting down. We are afraid of bone fractures so we avoid physical activities and falls as they are frightening. (…) She is being treated every 6 months at the hospital with a bone cancer treatment which has a lot of side effects during 5 days. She is in a frustrated state, with fever, pain to touch, she can't move and is incontinent*" (mother of a 40 years-old PMM2-CDG patient). In another experience, having osteopenia/osteoporosis limits the management of other clinical manifestations: "*due to osteopenia, he can't have surgery of the scoliosis because of the bone fragility. He cares about it* [scoliosis] *when he is in the wheelchair because it is very noticeable. There is not enough space on his body for the intestines and his lungs and sometimes he has very fast and short breathing*" (mother of a 25 years-old PMM2-CDG patient). This guided the QoL tools selection by making sure osteopenia/osteoporosis specific tools included items referring to pain, fear of fractures/falls, self-image, impact in care or treatment impact.

5.3.3. Review of the literature results according to the community-selected symptoms real-world qualitative information

The review of the literature concerning the application of PROMs specific for the community-selected impactful PMM2-CDG symptoms/manifestations resulted in the inclusion of 239 articles (Figure 5.1). The characteristics of the included articles are summarized in Table 5.2.

Table 5.2. Summary of the characteristics of included articles. Note: the sum of the percentages might not yield 100% due to numerical rounding.

Article summary	N	%
Number of patients		
≤ 100	136	56.9
101 to 500	73	30.5
≥ 501	30	12.6
Age Range		
Paediatric (< 18)	23	9.6
Adult (≥ 18)	187	78.2
Both	29	12.1
Type of QoL report		
Self-reported	226	94.6
Proxy-reported	4	1.7
Both	9	3.8
Disease classification (ICD-11)		
Neoplasms	7	2.9
Diseases of the blood or blood-forming organs	4	1.7
Diseases of the circulatory system	60	25.1
Diseases of the immune system	2	0.8
Endocrine, nutritional, or metabolic diseases	5	2.1
Sleep-wake disorders	1	0.4
Diseases of the nervous system	18	7.4
Diseases of the visual system	58	24.3
Diseases of the respiratory system	3	1.3
Diseases of the digestive system	58	24.3
Diseases of the musculoskeletal system or connective tissue	15	6.3
Diseases of the urinary system	1	0.4
Developmental anomalies	1	0.4
Symptoms, signs, or clinical findings, not elsewhere classified	6	2.5
Total number of included articles:	239	

Most articles (58.1 %) included small participant cohorts of ≤ 100 participants. While 29.9 % reported cohorts of > 100 to < 500 participants, only 12 % of the studies reported more than > 500 participants. Studies of adult populations represent most of the included studies (78 %). Only 10 % of the included studies focused on paediatric populations and 12 % included both adult and paediatric populations. QoL self-reports were described by most studies (94.2 %) whilst proxy-reports or a combination of both accounted for 2.1 % and 3.7 % of the studies, respectively.

Among the included articles, 14 disease groups were represented. Particularly prevalent in our study sample were diseases of the digestive, visual, and circulatory system followed by diseases of the musculoskeletal system, the connective tissue, and the nervous system.

The review of the included articles allowed the identification of 80 tools. These tools were grouped by signs/symptoms in Table 5.3. The list of references supporting the inclusion of such tools can be found in Supplementary Table 5.4. From the 22 groups of signs and symptoms, specific QoL tools were found for 15 of them (Table 5.3). No specific tools were found for 7 of the most impactful clinical manifestations, particularly for developmental delay, intellectual disability, hypotonia, pericardial effusion, peripheral neuropathy, stroke-like episodes, or symptoms related to deficient sexual development. However, even though no specific instruments were found for behaviour, developmental or intellectual problems, other included tools specific for other symptoms/diseases include subscales or items specific for those areas (e.g., mood swings, depression, physical, mental, and social functioning, etc.).

Table 5.3. Symptom/disease-specific quality of life tools per impactful symptom/category.

Category	Instrument name	Subscale/Domains	Nº items	Target Group (age, years)	Diseases assessed	Ref.
Ataxia	Quality of Life in Essential Tremor Questionnaire (QUEST)	Communication, work and finances, hobbies and leisure, physical, psychosocial	30	≥ 18	Essential tremor	[17]
	Multiple System Atrophy Quality of Life questionnaire (MSA-QoL)	Motor, nonmotor, emotional/social functioning	40	≥ 18	Multiple system atrophy	[18]
Cardiomyopathy	Kansas City Cardiomyopathy Questionnaire (KCCQ-12)	Physical limitation, symptom frequency, quality of life, and social limitation.	12	No reference	Cardiomyopathy	[19]
	Minnesota Living with Heart Failure Questionnaire (MLHFQ)	Physical impairment, emotional impairment	21	No specification ("elderly")	Heart failure	[20], [21]
Coagulopathy	Haemophilia-specific health-related quality of life questionnaire for adults (HAEMO-QoL-A)	Physical functioning, role functioning, worry, consequences of bleeding, emotional impact, treatment concerns	41	≥ 18	Haemophilia	[22]
	Haemophilia-specific health-related quality of life questionnaire - short form (HAEMO-QoL-SF)	Physical health, feelings, view of yourself, family, friends, other people, sports & school, dealing with haemophilia and treatment	35	4-17 (4-7; 8-17)	Haemophilia	[23]
	Canadian Haemophilia Outcomes-Kids' Life Assessment Tool (CHO-KLAT)	Treatment, physical health, family, future, feelings, understanding of haemophilia, other people and friends, and control over your life	35	4-18	Haemophilia	[24]
Dysarthria	Quality of Life in the Dysarthric Speaker (QOL-Dys)	Speech characteristics of the word, situational difficulty, compensatory strategies, perceived reactions of others	40	≥ 18	Dysarthria	[25], [26]
	Living with Neurologically Based Speech Difficulties (LwD)	Communication problems related primarily to speech, communication problems related primarily to language/cognition, communication problems related primarily to fatigue, effects of emotions, effects of different persons, effects of different situations, consequences of my difficulties in	50	≥ 18	Parkinson disease	[27]

		communicating, what contributes to the changes in the ways I communicate, communicating like I would want to, how do I perceive changes and the possibility to alter my ways of speaking				
	Dysarthria impact profile (DIP)	The effects of dysarthria on me as a person; accepting my dysarthria; how i feel others react to my speech; how my dysarthria affects my communication with others	52	No reference	Dysarthria acquired through different diseases (e.g. multiple sclerosis, motor neuron discase, Parkinson disease, stroke, Friedreich ataxia)	[28]
	Speech Handicap Index (SHI)	Psychosocial function; speech function	30	No reference	Oral and pharyngeal cancer	[29]
Dysphagia	Swallowing Quality Of Life (SWAL-QOL)	Burden, eating duration, eating desire, food selection, communication, fear, mental health, social, fatigue, and sleep	44	≥ 18	Friedreich ataxia; laryngotracheal disease; dysphagia	[30]–[32]
	Deglutition Handicap Index (DHI)	Physical, functional and emotional	30	≥ 18	Dysphagia	[33], [34]
	Eating Assessment Tool (EAT-10)	Unidimensional	10	≥ 18	Dysphagia	[35], [36]
	Dysphagia Handicap Index (DHI)	Physical, emotional, and functional problems	25	≥ 18	Dysphagia	[37]
Food Allergy	Food Allergy Quality of Life Questionnaire - Parent Form (FAQLQ-PF)	Emotional impact, food anxiety, social/dietary limitations	30	0-12 (0–3; 4–6; 7–12)	Food allergy	[38]
	Celiac Disease-specific DUX questionnaire (CDDUX)	Communication, having celiac disease, scale diet	12	8-18 (or their caregivers)	Celiac disease	[39]
Gastrointestinal (GI) problems	Short Inflammatory Bowel Disease Questionnaire (SIBDQ)	Bowel symptoms, systemic symptoms, emotional functioning, social functioning	10	≥ 18	IBD	[40]
	Inflammatory Bowel Disease Questionnaire (IBDQ)-9	Total score	9	≥ 18	IBD	[41]
	Irritable Bowel Syndrome Quality of Life Questionnaire (IBSQoL)	Emotional health, mental health, health belief, sleep, energy, physical functioning, diet, social role, physical role, and sexual relations	30	≥ 18	Irritable bowel syndrome	[42]
	Irritable Bowel Syndrome Quality of Life (IBS-QOL)	Dysphoria, interference with activity, body image, health worry, food avoidance, social reaction, sexual, relationships	34	≥ 18	Irritable bowel syndrome, chronic functional constipation, IBD	[43], [44]

Gastroesophageal Reflux Disease-Health Related Quality of Life (GERD-HRQL)	Total score	10 + 1 not scored	No reference	Gastroesophageal reflux disease	[45]
GastroIntestinal Quality of Life Index (GIQLI)	Core symptoms, physical items, psychological items, social items, disease-specific items	36	≥ 18	GI problems; type 2 diabetes mellitus; severe obesity; post-Whipple surgery and postcholecystectomy	[46]
Scleroderma gastrointestinal tract 1.0 questionnaire (SSC-GIT 1.0)	Reflux/indigestion, diarrhoea, constipation, pain, emotional well-being, social functioning	52	≥ 18	Systemic sclerosis	[47]
EORTC QLQ - Oesophageal Module 18 (OES18)	Dysphagia, eating, reflux, and pain plus 6 single items (swallowing saliva, choking, dry mouth, taste, cough, speech)	18	≥ 18	Dysphagia, esophageal eosinophilia, esophageal cancer	[48]
EORTC QLQ- Gastrointestinal Neuroendocrine Carninoid Module (GINET21)	Endocrine symptoms, GI symptoms, treatment-related symptoms, social functioning and disease-related worries plus 4 single-items (muscle and/or bone pain, body image, information and sexual functioning)	21	≥ 18	Gastroenteropancreatic neuroendocrine tumours	[49], [50]
Home parenteral nutrition-quality of life (HPN-QOL)	General health, ability to holiday or travel, coping, physical function, ability to eat and drink, employment, sexual function, and emotional function, body image, immobility, fatigue, sleep pattern, GI symptoms, other pain, presence or absence of a stoma, financial issues, and weight.	47	≥ 17	Chronic intestinal failure patients treated with home parenteral nutrition	[51], [52]
Izumo scale for abdominal symptom-related QOL	Heartburn, gastralgia, postprandial fullness, constipation, diarrhoea	15	No reference	Type 2 diabetes mellitus; upper GI symptoms, bone resorption and back pain in postmenopausal osteoporosis patients	[53]
Short Form-Nepean Dyspepsia Index (SF-NDI)	Interference with daily activities, knowledge/control, tension, work/study and eating/drinking	10	≥ 18	Irritable bowel syndrome	[54]
IBDQ-32	Bowel symptoms, systemic symptoms, emotional function and social function	32	≥ 18	Irritable bowel syndrome, irritable pouch syndrome	[55]

					and IBD (Crohn disease and ulcerative colitis)	
	IBDQ-36	Bowel symptoms, systemic symptoms, functional impairment, emotional functioning, social impairment	36	≥ 18	IBD	[56]
Infections	Sino-Nasal Outcome Test (SNOT-22)	Rhinologic, extra-nasal rhinologic, and ear/facial symptoms, psychological and sleep dysfunction	22	≥ 16	Chronic rhinosinusitis	[57]
	Sino-Nasal Outcome Test (SNOT-20)	Rhinologic, extra-nasal rhinologic, and ear/facial symptoms, psychological and sleep dysfunction	20	≥ 18	Bronchiectasis, allergic fungal rhinosinusitis	[58]
	Leicester Cough Questionnaire (LCQ)	Physical, psychological, and social	19	≥ 18	Bronchiectasis	[59]
Kyphosis/Scoliosis	Scoliosis Research Society (SRS-30)	Function/activity, pain, self-image/appearance, mental health, satisfaction with management	31	13-17, 18-64	Scoliosis	[60]
	SRS-22; SRS-22r	Function/activity, pain, self-image/appearance, mental health, satisfaction with management	22	13-17; 18-64	Adolescent idiopathic scoliosis	[61], [62]
	SRS-7	Unidimensional	7	13-18	Adolescent idiopathic scoliosis	[63]
	Early-Onset Scoliosis Questionnaire (EOSQ-24)	General health, pain/discomfort, pulmonary function, transfer, physical function, daily living, fatigue/energy level, emotion, parental impact, financial impact, satisfaction	24	0-18	Early-onset scoliosis	[64]
	Scoliosis Quality of Life Index (SQLI)	Physical activity performance, back pain, self-esteem, moods & feelings, and satisfaction with management	22	10-18	Adolescent idiopathic scoliosis	[65]
	The Italian Spine Youth Quality of Life questionnaire (ISYQOL)	Unidimensional	20	10-18	Adolescent idiopathic scoliosis	[66], [67]
Liver problems	Chronic Liver Disease Questionnaire for NAFLD and NASH (CLDQ NAFLD/NASH)	Abdominal symptoms, activity/energy, emotional health, fatigue, systemic symptoms, and worry.	36	≥ 18	Non-alcoholic steatohepatitis	[68], [69]
	Chronic Liver Disease Questionnaire (CLDQ)	Fatigue, activity, emotional function, abdominal symptoms, systemic symptoms, and worry	29	≥ 18	Cirrhosis, hepatic encephalopathy, cholestatic liver diseases	[70]

Ophthalmological problems	National Eye Institute Visual Functioning Questionnaire - 25 (NEI VFQ-25)	General vision, near vision, distance vision, driving, peripheral vision, colour vision, ocular pain, general health, and vision-specific role difficulties, dependency, social function, and mental health	25	≥ 18	RLBP1 retinitis pigmentosa, Friedreich's ataxia, myopia, hyperopia, astigmatism, nystagmus, strabismus, spinocerebellar ataxia, glaucoma	[71]
	National Eye Institute Refractive Error Quality of Life Instrument - 42 (NEI RQL-42)	Clarity of vision, expectations, near vision, far vision, diurnal fluctuations, activity limitations, glare, symptoms, dependence on correction, worry, suboptimal correction, appearance, and satisfaction with correction	42	≥ 18	Myopia, astigmatism, hyperopia	[72]
	VFS-plus	Unidimensional	19	≥ 18	Retinitis pigmentosa	[73]
	Pediatric Eye Questionnaire (PedEyeQ)	Functional vision, bothered by eyes/vision, social, frustration/worry, eye care	39-42	< 18 (0-4; 5-11; 12-17)	Strabismus and anisometropia	[74]
	Intermittent Exotropia Questionnaire (IXTQ)	Unidimensional	12	5-17 (5-7; 8-17)	Intermittent exotropia, strabismus, children wearing spectacles	[75]
	Adult Strabismus Quality of Life Questionnaire (AS-20)	Psychosocial and function	20	≥ 18	Epiretinal membrane, nondiplopic childhood-onset strabismus, strabismus, glaucoma, diplopia	[76]
	Adult Strabismus Quality of Life Questionnaire - 11 item (AS-11)	Unidimensional	11	≥ 18	Strabismus	[77]
	Low Luminance Questionnaire (LLQ)	Driving, extreme lighting, mobility, emotional distress, general dim lighting, peripheral vision	32	≥ 18	RLBP1 Retinitis Pigmentosa	[78]
	Questionnaire on the impact of strabismus on patient quality of life based on AS-20 from Ribeiro Gde B et al., 2014	Psychosocial aspects and functional aspects	20	> 7	Strabismus	[79]
	College of Optometrists in Vision Development Quality of Life questionnaire (COVD QOL)	Somatic, physical/occupational, social, and psychological	30	> 7	Intermittent central suppression of vision	[80]

	8-question QoL live interview developed by Kothari M et al., 2009	No reference	8	4-16	Strabismus	[81]
	Vision Quality of Life Questionnaire by McKeon C et al., 1997	Psychological well-being, perception of health, role functioning, physical health, visual function	32	8-46	Intermittent exotropia	[82]
	Questionnaire for Evaluating Quality of Life of Pathologic Myopia Patients by Takashima T et al., 2001	Vision-related daily tasks, social handicaps, emotional handicaps, leisure and support, cognition about disease, general well-being schedule, eye satisfaction, life satisfaction	52	≥ 18	Myopia	[83]
	Amblyopia and Strabismus Questionnaire (A&SQ)	Distance estimation, visual disorientation, fear of losing the better eye, diplopia, and social contact and cosmetic problems	26	≥ 18	Amblyopia, small-angle diplopia, strabismus	[84]
	Quality of Life Impact of Refractive Correction (QIRC)	Visual function, health concerns, well-being, convenience issues, symptoms, economic issues and cognitive issues	20	≥ 16	Myopia	[85]
	10-item Neuro-Ophthalmic Supplement (NOS) to the NEI-VFQ-25	Visual function and eye/lid appearance	10	≥ 18	Spinocerebellar ataxia	[86]
	HRQOL questionnaire by Chen et al., 2015	Visual function, psychosocial impact, social interaction and worries about vision	16	7-12	Amblyopia	[87]
	Disease specific questionnaire based on the study of the Joint LASIK Study Task Force (Schallhorn et al., 2016)	Patient satisfaction, effect of vision on various activities, ocular discomfort and visual phenomena	23	≥ 18	Post laser in situ keratomileusis intervention	[88]–[90]
	Nystagmus-specific QOL questionnaire (NYS-29)	Personal and social functional and physical and environmental functional	29	≥ 18	Nystagmus	[91]
	Quality-of-Life Scale for Myopia by Erickson et al., 2004	Tolerance of compromise/symptom, psychological states, frequency of compromise/symptom, extraversion/introversion, cosmesis	52	≥ 18	Myopia	[92]
Osteopenia	Short Osteoporosis Quality of Life Questionnaire (ECOS-16)	Physical functioning, illness-related fears, psychosocial functioning, and pain	16	≥ 18	Postmenopausal osteoporosis	[93], [94]
	Osteoporosis Assessment Questionnaire - Short Version (OPAQ)	Physical function, emotional status, symptoms and social interaction	34	≥ 18	Postmenopausal osteoporosis	[95]
	Osteoporosis-Targeted Quality of Life (OPTQoL)	Physical difficulty, adaptations and fears	32	≥ 18	Female osteoporosis	[96]

	Osteoporosis Quality of Life Questionnaire (OQLQ)	Symptoms, physical function, activities of daily living, emotional function and leisure	30	≥ 18	Female osteoporosis	[97]
	Mini-Osteoporosis Quality of Life Questionnaire (Mini-OQLQ)	Symptoms, physical function, activities of daily living, emotional function and leisure	10	≥ 18	Postmenopausal osteoporosis	[98]
	Quality of Life in Osteoporosis (Qualiost)	Physical and emotional	23	≥ 18	Osteoporosis	[99]
Seizures	Epilepsy & Learning Disabilities Quality of Life Questionnaire (ELDQOL)	Behaviour, seizure activity, mood and side effects	70	0-18	Epilepsy and learning disabilities, Dravet syndrome	[100], [101]
	Quality Of Life In Epilepsy (QOLIE-10/QOLIE-10-P)	Epilepsy effects, mental health, role function	10/11	≥ 18	Epilepsy	[102]
	Quality Of Life In Epilepsy (QOLIE-31)	Seizure worry, overall QOL, emotional wellbeing, energy/fatigue, cognitive functioning, medication effects, social functioning	31	≥ 18	Epilepsy	[103]
	Quality of Life in Epilepsy Inventory for Adolescents (QOLIE-AD-48)	Epilepsy impact, memory-concentration, attitudes towards epilepsy, physical function, stigma, social support, school behaviour, health perception	48	11-17	Epilepsy	[104]
	Quality of Life in Childhood Epilepsy Questionnaire (QOLCE)	Physical restrictions, energy/fatigue, attention/concentration, memory, language, other cognitive processes, depression, anxiety, control/helplessness, self-esteem, social interactions, social activities, stigma, behaviour, general health, and quality of life	91	4–18	Epilepsy	[105]
	Quality of Life in Childhood Epilepsy Questionnaire (QOLCE-55)	Cognitive, emotional, physical and social functioning	55	4-12	Epilepsy	[106]
Sleep disturbances	Insomnia Severity Index (ISI)	Initial (sleep-onset), middle (sleep maintenance), terminal (awakening), satisfaction, interference (with daily functioning), noticeability and distress	7	≥ 18	Insomnia	[107]
	Pittsburgh Insomnia Rating Scale (PIRS)-20	Nighttime and daytime distress symptoms, sleep parameters, quality, regularity, and depth of sleep	20	≥ 18	Diabetes mellitus type II and insomnia	[108]
Several signs/symptoms	Cushing QoL Questionnaire (CushQoL)	Daily life, emotional, and physical aspects	12	≥ 18	Cushing syndrome	[109]

	University of Washington Quality of Life Questionnaire (UW-QOL)	Pain, appearance, activity, recreation, swallowing, chewing, speech, shoulder, taste, saliva, mood and anxiety	12	≥ 18	Head and neck cancer	[110]
	University of California Los Angeles Prostate Cancer Index (UCLA-PCI)	Urinary, sexual and bowel function, urinary, sexual and bowel bother	20	≥ 18	Early state of prostate cancer	[111]

Legend: GI - Gastrointestinal; IBD - Inflammatory bowel disease

5.3.4. Quality assessment of included questionnaires

The quality of the 80 instruments was analysed using specific criteria from Terwee et al. (2007) (Supplementary File 5.1) [20]. Most instruments were evaluated with positive rating (+) for Content Validity (93.7 %), Construct Validity (77.5 %), Internal Consistency (71 %) and Reliability (60.8 %). For Agreement (73.4 %), Floor and Ceiling Effect (67.1 %), and Responsiveness (55.7 %), no sufficient information was found for most of the instruments. Lastly, regarding Criterion Validity and Interpretability analysis, the greater part of the information was unavailable (35.4 % and 24 %, respectively) or indeterminate (22.5 % and 59.4 %, respectively).

5.4. Discussion and future perspectives

Patient-centred outcomes have gained recognition in health technology assessment and clinical trial settings. Besides, they provide unique insights into the disease's natural history in terms of QoL and its fluctuations over time. Rather than just measuring clinically important outcomes, they offer the opportunity to access "patient-important" outcomes, meaningful to them when evaluating treatments or care [21]. For complex, chronic and/or rare diseases - with holistic challenges and for which the definition of disease biomarkers or clinical endpoints is puzzling - patient-reported QoL is of major importance providing a direct interpretation of the patient's response to treatment or care [22]. However, the scarcity of valid QoL PROMs and ObsROMs for most rare diseases and the challenges of validating the current available tools, pose a problem to adequately appraise potential treatments. Creative and pragmatic solutions are warranted to overcome difficulties related to small patient cohorts, the cost of tools' development, and the urgency for making new therapies available [4,5].

In this study, we applied an innovative methodology to accelerate the development of a PMM2-CDG-specific QoL questionnaire, while assuring its adequacy and meaningfulness by including the views and experiences of families and medical experts. By including both stakeholders' quantitative and qualitative input in the design of our literature search, we identified QoL instruments that matter the most. The differences in the perception of the most burdensome signs and symptoms between patients/caregivers and clinicians underscore patient/caregiver engagement and participation in all stages of the development of PROMs or ObsROMs as the only way to safeguard the relevance, adequacy, and comprehensibility of these tools [23]. However, particularly in rare, heterogeneous diseases, complementing the individual experience with the knowledge of medical experts is critical. While patients and family caregivers can highlight "hidden" aspects of the disease that are not clear or do not seem important to doctors, the latter can provide a wider perspective on the frequency, severity, and impact of clinical manifestations by studying patient cohorts. Furthermore,

clinicians will consider potential disease complications that patients may have not yet experienced. Importantly, conducting qualitative interviews complemented the quantitative results on "What is more important?" with "How and why is it more important?". In other words, listening to the description of the patient/family experience with illness (i.e., narrative medicine) provides meaning and understanding but also identifies the real impact of the disease outside clinics [24]. For CDG, this approach successfully reported the experiences of CDG parents, identifying major healthcare and educational needs [25]. This community-centric approach also allowed us to detect changes in the impact of clinical manifestations over time. Specifically, infections were shown to be burdensome in infancy but not in adulthood. Contrastingly, skeletal manifestations (kyphosis/scoliosis and osteopenia) did not pose a problem until later in life. Even though there are reports of these time-dependent clinical occurrences [26,27], there are no reports of their burden or impact on QoL for most clinical manifestations. Some pioneer attempts using patient-reported data were made to evaluate the impact of certain manifestations; however, they lacked the use of solid and validated questionnaires for that purpose [26,28]. Our study responds to this gap by identifying the manifestations that families and experts prioritize across age ranges and by providing specific tools that can measure QoL related to those symptoms.

Our quantitative results highlight that both stakeholder groups (families and professionals) rated neurological signs as the most impactful across all age ranges, particularly hypotonia, developmental delay, ataxia, dysarthria, and intellectual disability. This was corroborated by the qualitative interviews since these are manifestations that impact all domains of QoL (physical, social, and mental functioning as well as the capacity to perform daily living activities) throughout the patients' lives. Of note, these are also some of the most frequent clinical signs in PMM2-CDG patients [13]. Other neurological occurrences were also prioritized with lower impact scores, namely seizures and stroke-like episodes. These are mainly rare clinical events reported to happen in 13% and 7% of patients, respectively, but have been described as some of the most QoL-impacting issues in PMM2-CDG [13,29,30]. Surprisingly, much more pronounced impact scores are suggested by clinicians compared to families concerning these neurological manifestations. Considering the low frequency of these clinical signs, it is probable that the impact of these manifestations is underrated due to clinical representation bias. In fact, only 9/23 (39 %) of families reported any kind of QoL impact (from mild to extreme) derived from stroke-like episodes at some point of the patient's lives. Even though this percentage is still higher than the reported frequency, it might explain the low impact score from families since most of them rated stroke-like episodes as having no impact on their lives (IS = 1). Nevertheless, family-derived qualitative data indicates the physical, psychological, and mental burden attributed to these manifestations. Contrastingly, none of the seven family members interviewed were able to describe how peripheral neuropathy impacts their day-to-day life. Even though peripheral neuropathy was present in some of their clinical reports, the real and physical consequences (e.g., pain to touch, numbness, altered

sensations) were not perceived by the interviewed families, which might explain the differences of impact perception compared to medical professionals. These observations show the importance of complementing patient-reported data with medical knowledge and experience. Given the low number of interviews performed, further studies should secure bigger patient cohorts of worldwide and differently aged patients and representation of the full clinical spectrum and severity of PMM2-CDG to further understand the burden of the disease accurately.

Other system-related manifestations were prioritized concordantly by families and professionals, namely ophthalmologic manifestations, infections, overheating episodes, behavioural problems, kyphosis/scoliosis, and osteopenia. On the contrary, families and doctors rated dysphagia and sex development deficiencies differently. Since dysphagia is often a consequence of hypotonia, while doctors have this knowledge, families might not have had the opportunity to become familiar about the difference between "dysphagia" and "difficulty swallowing due to muscle weakness (hypotonia)". In fact, families rated hypotonia with high impact scores. A similar issue arises considering that food allergies were included as a QoL-impacting manifestation. Even though food allergies have been pointed out as having a negative impact in PMM2-CDG patients' QoL [26], interviewed families did not experience this clinical issue. Moreover, the medical committee pinpointed that food allergies are extremely rare in PMM2-CDG but food intolerances are rather common. This inconsistency raises the possibility that families consider food "allergy" and "intolerance" interchangeable terms. Therefore, efforts should be taken to improve the communication between the medical teams and families, raise health literacy levels and contribute to the proper disease understanding and management. An action that could be taken in the future to help manage and minimise these differences encompasses creating and distributing glossaries explaining medical and difficult terms in lay-language to empower families to participate confidently. This methodology has proven helpful and effective in other people-centric studies [26,31].

Some general patient-reported clinical assessment tools have already been used in clinics for PMM2-CDG, particularly the Goal Attainment Scale and the patient-centred measures from Patient-Reported Outcomes Measurement Information System (PROMIS) [32,33]. However, none of these reflect the most impactful conditions presented by the patients. Our methodology answered this gap and allowed us to tailor our search for adequate PRO tools for most of the included disease manifestations. Importantly, 94.2% of the articles reporting the use of the 80 QoL tools identified used them as self-reports. This is normally considered as the best practice since it does not require interpretation by a proxy [34]. However, most included articles also reported PROMs use in mono-organ and non-neurologic diseases, mostly allowing the use of self-reporting. In the case of PMM2-CDG, the cognitive and motor impairment, as well as communication difficulties due to dyspraxia will restrain most patients to self-report how they feel and function. Proxy assessments - a proxy responding to a QoL tool aimed for self-reporting as they believe the patient would rate the

items) - need to be put into place as a solution, as performed for other debilitating diseases [19,35]. Typically, proxy reports tend to overestimate the QoL impact compared to self-rating, but it might depend on several factors (e.g., QoL domain, disease severity or difficulty of carer's tasks). Nonetheless, in several instances, proxy-reports were found to correlate with self-assessments. Besides, we believe that the over or underestimation of QoL from proxies can be systematic and therefore, changes across time and following an intervention should be captured. Measuring clinical severity alongside proxy-reports might be a way to ensure their reliability [33]. However, we cannot discard that a minority of PMM2-CDG patients might be able to express themselves and provide QoL self-ratings. Different rating methods and creative tools should be available to ensure their inclusion.

We also aimed to objectively analyse the psychometric quality of the questionnaires, since they will be the basis for the development of a future PMM2-CDG QoL questionnaire. Our results showed that some psychometric properties are, in general, objectively calculated. Even so, for Construct Validity most articles did not explicitly present the hypotheses. This is a common and potential risk, as the instrument may not represent the intended construct [20]. On the contrary, most instruments showed a positive Content Validity rating, considered the most important psychometric property of a tool [36]. Since health-related questionnaires are essential to assess the impact of a disease or treatment, Agreement should be accessed in validation articles to define the absolute measurement error, required for evaluative purposes to distinguish clinically meaningful changes. However, properties such as Agreement, Floor and Ceiling effect, Responsiveness, Interpretability and Criterion Validity are rarely reported. Thus, future validations should include the assessment of these psychometric dimensions.

The recent advances in drug development programs and increase in clinical research for PMM2-CDG urge for a disease-appropriate and responsive HrQoL measure. This study is a step towards the development of this PMM2-CDG QoL questionnaire assuring the engagement and participation of families and doctors since its inception. Following efforts should adopt the same methodology complementing clinically important factors (doctors' views) with aspects that make life worth living (patients' and families' views). Looking forward, item selection from the gathered questionnaires following standard development procedures and item reduction should follow a programmed decision system including all stakeholders and resorting to nominal groups and cognitive interviews.

Limitations of this study

There were several limitations to this study. This is a pilot study that shows the potential of a community-centric methodology in PROMs development. Nevertheless, our results should be interpreted cautiously given the small sample participating in the impact survey and interviews. Even though our study aimed to include families representing different severities as well as different age ranges, the low number of families participating in the impact survey and the interviews are not representative of the full spectrum of PMM2-CDG clinical

presentation, severity, and heterogeneity. As an example, we reached very limited representation of families of adult patients (n=4 on the impact survey and n=1 in the qualitative interviews). Next efforts should be taken to increase patient representation and capture the huge variability of clinical presentation and PMM2-CDG. Additionally, both in the questionnaire and interview data on phenotypic severity should be collected to allow the stratification of the patient population according to disease severity and investigate if and how that affects HrQoL tool identification and, consequently PROMs development and/or administration.

We queried the PubMed database and no other sources because the project is led by a non-profit organisation without external funding. Non-English articles and articles using translated versions of the questionnaires were not included for practical reasons resulting in limited negative evidence. Although we are aware that we did not include all available instruments for the symptoms assessed, our methodology answered the main goal of this study - to identify the main questionnaires used across the impactful signs and symptoms. In our QoL tool quality analysis, the evaluation was centred on the criteria developed by Terwee et al., (2007), which are primarily opinion-based, and for which there is no empirical evidence to support explicit quality criteria in this field. However, it allowed us to establish a method of quality comparison between instruments. Also, there are some measurement properties that, despite being identified, are not assessed through the predefined criteria. Hence, there may be a need to "refine" or adapt these guidelines so that future comparative questionnaire analysis could be more accurate. Furthermore, the quality ratings do not consider a systematic review of validation studies associated with each questionnaire and depend on the availability of information on original development and validation articles. Finally, we cannot conclude that questionnaires with the highest number of positive ratings are necessarily the best ones, since some validation properties are more critical than others, depending on the aim of the questionnaire (e.g., discriminative questionnaires require a high level of reliability to be able to distinguish between people, while evaluative questionnaires require a high level of agreement to be able to measure essential changes). However, although important, this limitation did not interfere with the purpose of our analysis.

5.5. Conclusion

Accurately measuring HrQoL using a PMM2-CDG-specific QoL questionnaire including the most concerning domains/symptoms from both the family and medical perspectives will benefit therapy development and approval but also for establishing the natural history of the disease in terms of QoL. In turn, it will require and benefit from the combined efforts from all stakeholders, particularly families, researchers, clinicians, and pharma representatives as shown in this study. As for other rare diseases, creative and new approaches for the development of such scales need to be applied, particularly given the clinical heterogeneity of PMM2-CDG throughout time and between patients. This study provides a solution for this

matter particularly by surveying the patient and medical community about the most impactful symptoms through the lifespan of a patient providing a list of adequate tools/items for the development of a new specific scale.

5.6. References

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CHAPTER 6.

SIALYL LEWIS$^{X/A}$ AND CYTOKERATIN CROSSTALK IN TRIPLE NEGATIVE BREAST CANCER

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Abstract

Triple-negative breast cancer (TNBC) encompasses multiple entities and is generally highly aggressive and metastatic. We aimed to determine the clinical and biological relevance of sialyl-lewis X and A ($sLe^{X/A}$) - a fucosylated glycan involved in metastasis - in TNBC. Here, we studied tissues from 50 TNBC patients, transcripts from a TNBC dataset from The Cancer Genome Atlas (TCGA) database, and a primary breast cancer cell line. All 50 TNBC tissue samples analysed expressed $sLe^{X/A}$. Patients with high expression of $sLe^{X/A}$ had 3 years less disease-free survival than patients with lower expression. In tissue, $sLe^{X/A}$ negatively correlated with cytokeratins 5/6 (CK5/6, which was corroborated by the inverse correlation between fucosyltransferases and CK5/6 genes. Our observations were confirmed in vitro when inhibition of $sLe^{X/A}$ remarkably increased expression of CK5/6, followed by a decreased proliferation and invasion capacity. Among the reported glycoproteins bearing $sLe^{X/A}$ and based on the STRING tool, α6 integrin showed the highest interaction score with CK5/6. This is the first report on the $sLe^{X/A}$ expression in TNBC, highlighting its association with lower disease-free survival and its inverse crosstalk with CK5/6 with α6 integrin as a mediator. All in all, $sLe^{X/A}$ is critical for TNBC malignancy and a potential prognosis biomarker and therapeutic target.

Keywords: triple-negative breast cancer (TNBC); sialyl $Lewis^{X/A}$ ($sLe^{X/A}$); cytokeratin expression; intermediate filament proteins; disease-free survival rate; α6 integrin; aberrant glycosylation

6.1. Introduction

Breast cancer (BC) is notorious for its heterogeneity, making personalised medicine difficult to implement in clinical settings. Triple-negative breast cancer (TNBC) is one of the most aggressive types of BC, with a poor prognosis, advanced stage, and an increased risk of visceral or brain metastasis [1]. At the time of diagnosis, these tumours are typically larger in size, have a higher histological grade (III) and involve lymph nodes [2]. TNBC is distinguished from other types of BCs by the absence of expression of estrogen receptors, progesterone receptors and epidermal growth factor receptors type 2 (HER2), representing approximately 15% of all types of BCs [3]. TNBC is mainly treated with chemotherapy because it does not respond to endocrine or anti-HER2 directed therapy and, as such, only a few targeted therapies have been approved [3]. In addition, TNBC's patients have shorter disease-free survival and overall survival compared to other BC patients. Noteworthy is that more than 70 % of women with metastatic TNBC do not survive more than five years after initial diagnosis [4].

Since TNBC encompasses multiple and heterogeneous entities with unique profiles, attempts to subtype TNBC have been intensively explored to implement biomarker-driven therapeutic approaches [5]. The most studied biomarkers which include the intermediate filament cytokeratin (CK)5/6, epidermal growth factor receptor (EGFR), P-cadherin and androgen receptor (AR), showed limited clinical value [6]–[8]. Furthermore, it is still unclear which mechanisms underpin TNBC and which factors influence its invasive and metastatic behaviour.

During tumour progression and metastasis, tumour cells initiate the epithelial-mesenchymal transition program, becoming motile cells capable of circulating to other organs [9]. In this process, the interaction of protein complexes called hemidesmosomes (HD) with the microenvironment determines the strength of tumour cells' adhesion to the extracellular matrix (ECM). These interactions are mediated by its composing HD adhesion molecules including integrin α6β4. When integrins are phosphorylated, the HD disassemble, allowing the intermediate cytoskeleton filaments to detach from the ECM and activate intracellular signalling pathways (e.g., EGFR/PI3K/Akt and FAK/Src), resulting in a more tumorigenic profile [10], [11].

While circulating in the bloodstream, tumour cells with the capacity to interact and bind to vascular endothelium can invade distant organs. This is accomplished by the binding of tumour cells to E-selectins expressed by endothelial cells. E-selectins are adhesion molecules required for leukocyte recruitment during the early stages of inflammation [12], [13]. They are induced by inflammatory cytokines and constitutively expressed in cancer and marrow microvasculature [12], [14]. When they bind to their ligands, they set off a chain of events that leads to the cell adhesion and subsequent transendothelial migration [15], [16]. E-selectins bind to sialofucosylated glycans, namely sialyl Lewis X (sLe^X) and sialyl Lewis A (sLe^A), displayed on cell surface proteins and acts as its major ligands [17], [18]. The protein scaffold modulates the presentation of $sLe^{X/A}$ and subsequent interaction with E-selectin and intracellular signalling [19], [20]. The overexpression of the E-selectin ligands (E-SL) is frequently observed

in cancer, mostly due to the increased activity of α1,3-fucosyltransferases that catalyse terminal fucosylation steps [21].

There are numerous reports on the elevated $sLe^{X/A}$ expression in BC, namely in tissues, cell lines, and serum [22]–[24]. Interestingly, its expression is higher in metastatic lesions than in primary tumour tissues, and in some cohorts, it correlates with poor prognosis [25], [26]. E-SL are also known to be expressed by TNBC [27], yet their relevance and the importance of $sLe^{X/A}$ has not been properly addressed.

In this study, we examined the expression of $sLe^{X/A}$ in TNBC tissue from a cohort of patients. It was observed that $sLe^{X/A}$ was expressed in all TNBC and associated with decreased disease-free survival. We also identified a negative correlation between the immunohistochemical expression of the $sLe^{X/A}$ and CK5/6. Moreover, inhibition of $sLe^{X/A}$ in a BC cell model increased the expression of CK5/6, and reduced cell proliferation and invasion capacity. In our cell model, $sLe^{X/A}$ decorates α6 integrin and its expression impacts the phosphorylation of the downstream intracellular signalling. Collectively, these findings unveil a molecular interplay between $sLe^{X/A}$ and intermediate filaments. It also suggests that this glycan may be exploited to enable better patient stratification and as a therapeutic target in TNBC.

6.2. Materials and Methods

6.2.1. Patients and Clinical Data

This study was performed after institutional ethical approval (Internal Reference Code – SBraga2014) and patient informed consent. It involved 50 TNBC patients who underwent surgery in Hospital CUF Descobertas (Lisboa, Portugal). For each patient, tissue specimens were fixed in formalin and embedded in paraffin. The determination of the molecular subtype was performed by the Hospital's pathology laboratory. A summary of the clinical data is available in Supplementary Table 6.1.

6.2.2. Immunohistochemical Staining of TNBC Sections

Paraffin-embedded sections of TNBC specimens were deparaffinised in trilogy pre-treatment solution (Cell Marque, Rocklin, CA, USA) at 94 °C and antigens recovered in Lab Vision PreTreatment Module (Thermo Scientific, Waltham, MA, USA). After blocking endogenous peroxidase (Atom Scientific, Hyde, UK), sections were stained with anti-CK5/6 (1:200; Cell Marque, Rocklin, CA, USA), anti- AR (1:100; Cell Marque, Rocklin, CA, USA), anti-P-cadherin (1:1000; Abcam, Cambridge, UK) or anti-EGFR (1:100; Abcam, Cambridge, UK) for 1h in "Diamond: Antibody Diluent" (Cell Marque, Rocklin, CA, USA). We used the previously described immunohistochemical staining protocol for $sLe^{X/A}$ and E-SL detection [27], [28]. $sLe^{X/A}$ was stained with the HECA-452 antibody clone (1:50, Biolegend, San Diego, CA, USA),

whilst E-SL were stained with E-Immunoglobulin (Ig) chimaera (1:100) followed by anti-Cluster of Differentiation (CD) 62E monoclonal antibody (mAb) (1:50; BD Biosciences, Franklin Lakes, NJ, USA) in Tris-Buffered Saline with Tween, which in case of E-Ig staining was supplemented with 2 mM CaCl2, as described [27]. All slides were stained with "HiDef Detection HRP Polymer System" (Cell Marque, Rocklin, CA, USA). The colour was developed using 3,3'-diaminobenzidine solution (ScyTek Laboratories, Logan, UT, USA). Subsequently, sections were stained with haematoxylin (Bio-Optica, Milan, Italy) for nuclear contrast staining before dehydration, clearing and mounting with Quick-D mounting medium. The slides were visualized under a light microscope with coupled camera by two certified independent pathologists. The localisation of the expression and the abundance of stained cells was scored using 4 categories: absence or very weak labelling (0); weak staining of less than 1/3 of the cells (1); moderate intensity staining of 2/3 of cells (2), and strong staining of 3/3 of the cells (3).

6.2.3. Cell Culture and Treatments

The human BC cell line, CF1_T, was obtained and immortalised with human telomerase reverse transcriptase cDNA transduction, as described previously [20], [28] [20,28]. It was cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with foetal bovine serum, glutamine, penicillin, and streptomycin in T25 flasks at 15% confluence. To reduce the expression of cell surface $sLe^{X/A}$ and E-SL, CF1_T cells were passed, concurrently treated for 5 days with 1 mM 2-fluorofucose (2-FF, Biosynth Carbosynth, Compton, UK) with a change to new 2-FF supplemented medium at the third day and then analysed.

6.2.4. Fluorescence Microscopy

Cells were cultured on round coverslips inside 24 well plates (Orange Scientific Braine-l'Alleud, Belgium) for 24 hours. Then cells were washed, fixed and permeabilised with 0.1% TritonX100 and blocked with 1% bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA). Cells were subjected to immunofluorescence staining with anti-$sLe^{X/A}$ (1:50; HECA-452 clone; Biolegend San Diego, CA, USA) and anti-CK5/6 (1:200; Cell Marque, Rocklin, CA, USA). Cells were incubated with anti-rat IgM-Fluorescein isothiocyanate (FITC) (1:50) (BD Pharmingen, San Diego, CA, USA) or anti-mouse IgG-FITC (1:100) (Dako, Santa Clara, CA, USA) secondary antibodies. Finally, nuclei were stained with 4',6-diamidino-2-phenylindole (1µg/mL, ThermoFisher, Waltham, MA, USA) for 10 minutes, washed, mounted with montage medium Mowiol+1,4-diazabicyclo[2.2.2]octan, and analysed. Fluorescence intensities from five randomly selected microscopic fields of cells were quantitatively analysed with ImageJ

software and using the corrected total cell fluorescence (CTCF) formula: CTCF = Integrated density – (Area of selected cell x Mean fluorescence of background readings).

6.2.5. Western Blot

Cells were lysed using Pierce® Immunoprecipitation Lysis buffer (ThermoFisher, Waltham, MA, USA) supplemented with cOmplete™, Mini, ethylenediaminetetraacetic acid (EDTA)-free Protease Inhibitor Cocktail (Roche, Basileia, Switzerland). The protein was quantified with the Pierce™ bicinchoninic acid Protein Assay kit (ThermoFisher, Waltham, MA, USA) following the manufacturer's instructions. An 8% acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel was loaded with 30 µg of denatured protein. After the run, proteins were transferred to a nitrocellulose membrane (Amersham™ Protran® Premium 0.45 µm NC, Amersham, UK) and blocked with Carbo-Free™ Blocking solution (VectorLabs, Newark, CA, USA). Incubation with a mouse anti-human plectin IgG1 primary antibody (SantaCruz Biotechnology, Dallas, TX, USA) or mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich, Saint Louis, MO, USA) was performed overnight at 4ºC. Secondary detection with the goat anti-mouse horseradish peroxidase antibody was performed for 1h at room temperature (BD Biosciences, Franklin Lakes, NJ, USA). Signal was revealed using the Lumi-Light Western Blotting Substrate (Roche, Basileia, Switzerland) and X-ray films (Amersham Hyperfilm™ ECL, Amersham, UK). Membrane stripping was achieved using the Restore™ Western Blot Stripping Buffer (ThermoFisher, Waltham, MA, USA). The western blot bands quantification was conducted using the ImageJ software v.1.53.

6.2.6. Flow Cytometry

To analyse the expression of intracellular markers by flow cytometry, cells were first permeabilised using Fixation/Permeabilization Solution Kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were stained with 1:1000 dilution of anti-phosphorylated (p)-Src (Tyr416), anti-total Src, anti-p-AKT (Ser473), anti-AKT1/2, anti-p-ERK1/2, anti-ERK1/2, anti-p-p38 MAPK and p38 MAPK mAbs, all from Cell Signaling Technology at 4 °C for 30 min. For cell surface α6 integrin staining, the anti-CD49f antibody was used (BD Biosciences, Franklin Lakes, NJ, USA). A FITC-conjugated anti-mouse IgG (Dako, Santa Clara, CA, USA) was used for secondary detection at 4ºC for 20 min. Cells were washed and fixed with 2% paraformaldehyde. Data were acquired using the Attune Acoustic Focusing Cytometer (Applied Biosystems, Waltham, MA, USA) and analysed with FlowJo software version 10.0.5 (BD Biosciences, Franklin Lakes, NJ, USA). Data were presented as delta mean fluorescent intensity (MFI) obtained by subtracting the MFI of the secondary staining control.

6.2.7. TCGA Analysis

The data from TNBC patients, namely clinical metadata (e.g., patient, tumour stage, follow-up information) as well as RNA-Seq read counts in the form of HTSeq count tables were obtained from various breast invasive carcinoma cases using the 'brca.data' R package and pipeline previously described by Lopes et al [29]. Briefly, clinical data on ER, PR, and HER2 expression were used to select TNBC cases. TNBC patients were considered when all three markers were "negative". A final dataset of TNBC patients with logarithmically transformed (base 2) transcripts per million was created to access gene expression.

TNBC samples were divided by their high or low gene expression for each of the fucosyltransferases (FUT) gene expressions (*FUT3, 4, 5, 6, 7* and *10*) based on the median of all samples. The expression of the genes that code for CK5/6 (*KRT5, KRT6A, -6B, -6C*) was compared between the high and low FUT expression groups.

6.2.8. Statistical Analysis

The R v 4.1.1 computational language [30] was used for the following statistical analyses and the scripts made available as Supplementary File 6.2, 6.3 and 6.4. The disease-free survival (DFS) was estimated using the Kaplan-Meier approach and compared between groups using the log-rank test employing the survival (v3.4-0) [31] and the survminer (v0.4.9) R packages. We used the Shapiro–Wilk normality test to determine the normality of variables. The non-parametric Spearman correlation was used to examine the relationship between the data. Using the GraphPad Prism 8.4 (GraphPad Software, Inc, San Diego, CA, USA), the unpaired t-tests were performed to compare E-SL/sLe$^{X/A}$ expression between the high and low CK5/6 expression groups and cell signalling proteins' expression between 2-FF treated and non-treated cells. An unpaired student's t-test with Welch's correction was used to compare CK genes expression between the high and low FUTs expression. Tests were considered statistically significant when $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) or marginally significant when $0.05<p<0.1$. All bar plots were created using GraphPad Prism 8.4.

6.3. Results

6.3.1. Biomarker characterisation and correlation with clinical features

TNBCs are generally characterised by poor prognosis and heterogeneous biology. In this study, the median DFS of our TNBC patient cohort was 802.5 days (Min= 89 days, Max= 1826 days) with a 50% probability of recurrence after approximately 2 years and 5 months (Figure 6.1A). Most tumours were high grade (56% grade III and 2% grade IV) and poorly differentiated, whilst 26% were of moderate grade (grade II) and 16% were low grade (grade I). The median patient's age at diagnosis was 64 years (Min= 33 years; Max= 89 years). The

median tumour size was 15 mm (Min= 2 mm; Max= 70 mm) and the median number of invaded sentinel lymph nodes by tumour cells was 12 (Min= 1; Max= 25). The clinicopathologic characteristics of this TNBC cohort is shown in Supplementary Table 6.1.

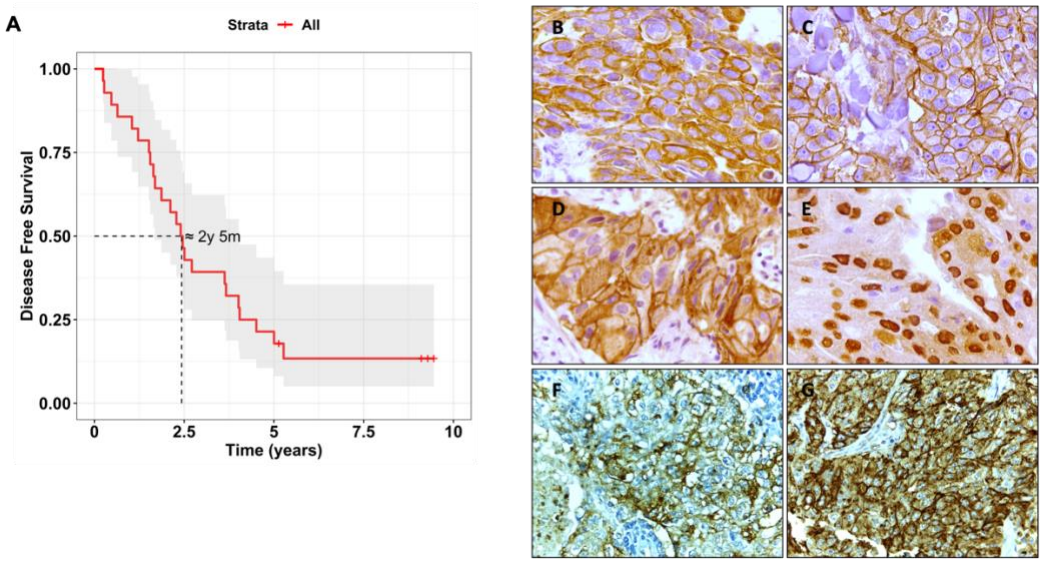

Figure 6.1. Disease-free survival (DFS) of the triple-negative breast cancer (TNBC) patient cohort and respective immunohistochemical biomarker staining of respective TNBC tissues. (A) Kaplan-Meier curve of DFS of all TNBC patients enrolled in this study generated with the survminer (v0.4.9) and survival (v3.4-0) R packages. Representative microphotographs (400X magnification) of immunohistochemical staining against cytokeratin (CK) 5/6 (B), P-cadherin (C), Epidermal Growth Factor Receptor (EGFR) (D), Androgen Receptor (AR) (E), Sialyl-Lewis X and A ($sLe^{X/A}$) (F) and E-selectin ligand (E-SL) (G) in TNBC tissue sections. Tissues were stained with haematoxylin which colours nuclei. The staining with antibodies or E-Ig was followed by a horseradish peroxidase conjugated secondary antibody and visualised in brown.

To characterise the molecular profile of the TNBC patient cohort, the expression of the biomarkers CK5/6, EGFR, P-cadherin, AR, $sLe^{X/A}$ and E-SL were measured in tumour sections by immunohistochemistry (Figure 6.1, Table 6.1). Staining of CK5/6 expression was observed in the cytoplasm (Figure 6.1B). Its expression was observed weakly in 24% of the cases, moderately in 12% and strongly in 8% of the cases. P-cadherin staining was observed mostly in the membrane in 96% of the cases (Figure 6.1C, Table 6.1). EGFR was detected in the cell membrane very strongly in 14% of the cases, while 60% of cases had weak or very weak staining, and 26% had moderate staining (Figure 6.1D, Table 6.1). AR staining was mostly nuclear and positive in 34% of the cases (Figure 6.1E, Table 6.1). $sLe^{X/A}$ was expressed in all cases, with 30 % being strongly expressed while moderately and weakly expressed in 30 and 40% of the cases, respectively. $sLe^{X/A}$ staining was identified mostly both in the cell membrane and cytoplasm (65%), while 15% of the cases presented only cytoplasmic staining, and 20% only cell membrane staining (Figure 6.1F, Table 6.1). E-SL was also expressed in all cases, being strongly expressed in 55% of the cases, while weakly expressed in 20% and moderately expressed in 25% of the cases. In 60% of the cases, E-SL staining was found in cytoplasm and

plasma membranes. In 25% of the cases, E-SL presented just cytoplasmic staining, while 15% had only on-cell membrane staining (Figure 6.1G, Table 6.1).

Table 6.1. Distribution of the expression of the biomarkers in the TNBC population tested in this study.

Biomarker	Biomarker staining[1]	% of cases
CK5/6	0	56%
	1	24%
	2	12%
	3	8%
EGFR	0	30%
	1	30%
	2	26%
	3	14%
AR	Negative	66%
	Positive	34%
P-cadherin	Negative	4%
	Positive	96%
$sLe^{X/A}$	0	0%
	1	40%
	2	30%
	3	30%
E-SL	0	0%
	1	20%
	2	25%
	3	55%

[1]The staining score consists in 0- absence or weak labelling; 1- weak staining of less than 1/3 of the cells; 2- moderate intensity staining of 2/3 of cells and 3- strong staining of 3/3 of the cells.

6.3.2. $sLe^{X/A}$ and E-SL expression negatively correlate with CK5/6 expression in TNBC

We then assessed for correlations between $sLe^{X/A}$ and E-SL; clinicopathologic features and molecular profile. $sLe^{X/A}$ and E-SL reactivity showed a significant and positive correlation ($r = 0.660$, $p = 0.002$, Figure 6.2A). Comparing the expression of these two markers with the expression of the other analysed molecules, we verified that $sLe^{X/A}$ expression was negatively correlated with CK5/6 expression ($r = -0.516$; $p = 0.019$). Similarly, total E-SL expression also tended to be negatively correlated with the expression of CK5/6 ($r = -0.443$; $p = 0.051$). Comparing the expression of $sLe^{X/A}$ and total E-SL between the negative (expression = 0) and

positive (expression ≥ 1) CK5/6 cases, we verified that both markers are significantly more expressed in negative CK5/6 cases compared to the positive ones (Figure 6.2B, C).

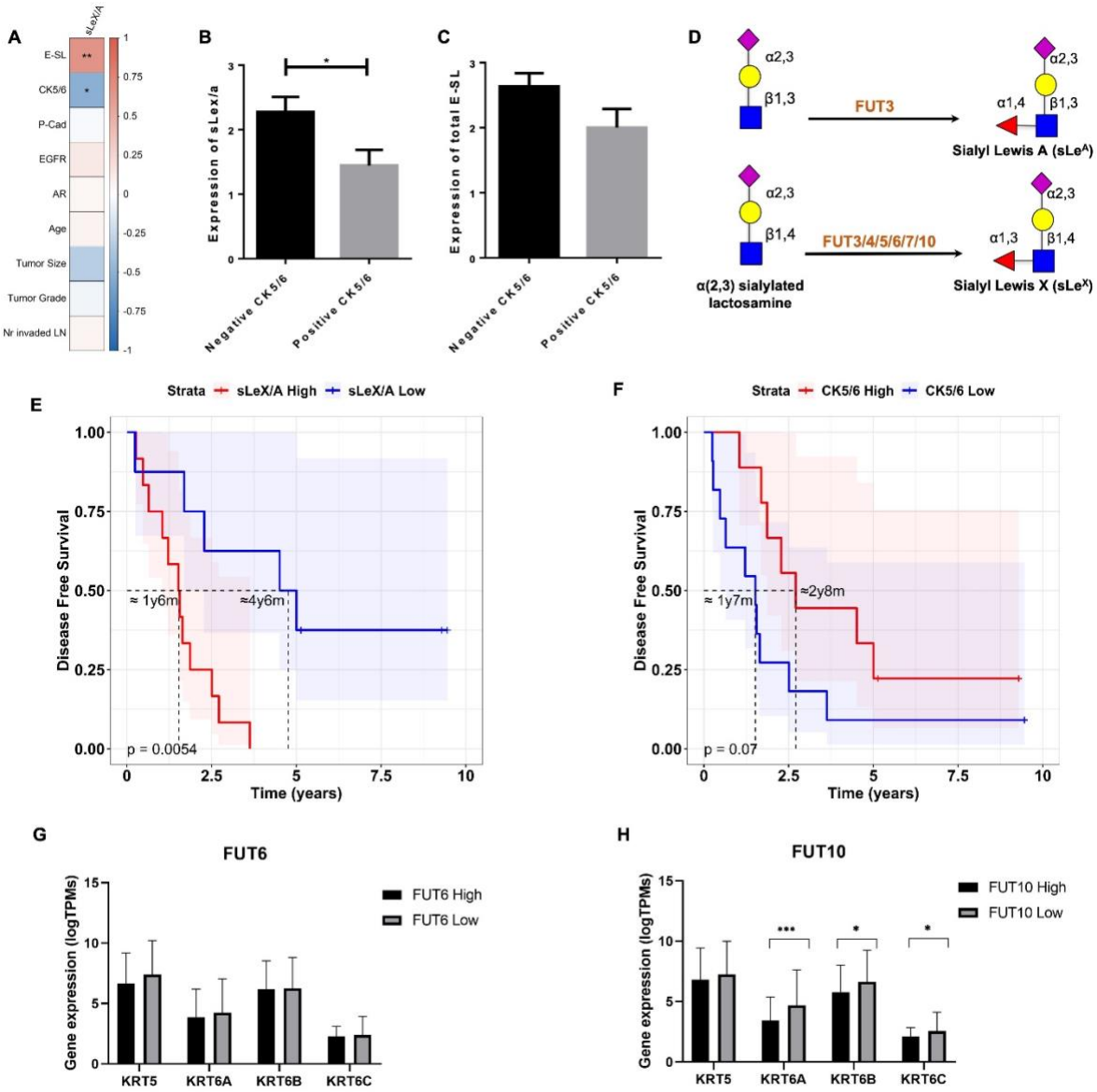

Figure 6.2. $sLe^{X/A}$/E-SL and CK5/6 expression in TNBC tissues are inversely correlated and influence DFS. (A) $sLe^{X/A}$ is positively correlated with E-SL staining (p = 0.002) and negatively correlated with CK5/6 expression (p = 0.019) in TNBC tissues. Non-parametric spearman correlation was used to evaluate the association between features. CK5/6-positive TNBC have lower expression of $sLe^{X/A}$ (B) and E-SL (C) than the TNBC samples that do not express the CK5/6. TNBC cases were divided in two groups according to CK5/6 expression (score ≥ 1) or lack of expression (score = 0); (D) sLe^{X} and sLe^{A} structure and terminal step of addition of fucose by fucosyltransferases (FUT). (E) Patients with lower $sLe^{X/A}$ expression have a better 10-year disease-free survival than those with higher expression (p = 0.0054); (F) Contrastingly, patients with lower CK5/6 expression have worse 10-year DFS than those with higher expression (p = 0.0696); Kaplan-Meier curves show the DFS of patients with high (red) and low (blue) $sLe^{X/A}$ or CK5/6 expression (higher and lower expression values than the mean $sLe^{X/A}$/CK5/6 expression, respectively) generated with the survminer (v0.4-9) and survival (v3.4-0) R packages. (G) FUT6 low gene expression group has increased KRT6A gene expression (p = 0.074), and (H) FUT10 low gene expression group has increased KRT6A (p = 0.002), -6B (p = 0.034) and -6C (p = 0.025), compared with the high expression groups. TNBC tissue genetic data from the TCGA database was analysed for gene expression. Samples were subdivided based on the median expression of *FUT6* and *FUT10* and correlated with genes involved in CK5/6 epitope expression (*KRT5, KRT6A, -6B, -6C*). Legend: * - p < 0.05, ** - p < 0.01, *** - p < 0.001.

Interestingly, regarding the influence of $sLe^{X/A}$ expression, we found that patients with low expression of $sLe^{X/A}$ had significantly higher DFS (p = 0.005) and only reached a 50% probability of disease recurrence after 4 years and 6 months when comparing with patients with high expression of $sLe^{X/A}$ that reached the same probability after 1 year and 6 months (Figure 6.2E). Further corroborating the negative correlation between $sLe^{X/A}$ and CK5/6, high expression of CK5/6 shows a tendency to higher DFS (marginally significant, p = 0.0696), with a 50% DFS probability at approximately 2 years and 8 months, compared to a 1 year and 7 months in the CK5/6 low expression group (Figure 6.2F). This relationship with the DFS is almost the inverse of what it is seen for the $sLe^{X/A}$ (Figure 6.2E), reinforcing the negative correlation between these two biomarkers. To further corroborate the inverse correlation between $sLe^{X/A}$/ E-SL and CK5/6 expression, we analysed the gene expression of potential key players in this interaction from 160 TNBC patients publicly available in TCGA. The data retrieved concerned the gene expression of the fucosyltransferases (enzymes responsible for completing the $sLe^{X/A}$ structure during their biosynthesis [32], Figure 6.2D), namely *FUT3, 4, 5, 6, 7* and *10*, and of the genes responsible for the synthesis of the CK5/6 biomarker epitope, namely *KRT 5, 6A, 6B, 6C*. Supporting our previous observations, we noted that when subdividing TNBC patients into a high or low expression of the fucosyltransferase *FUT6* and of *FUT10*, the groups with lower expression of *FUT6/10* have higher expression of CK6 genes (Figure 6.2G, H, Supplementary Figure 6.1).

Altogether, the data suggest that in TNBC tissues, the higher phenotypic expression of $sLe^{X/A}$ leads to a CK5/6 decrease and vice versa. The data also suggests that the expression of the genes underlying the $sLe^{X/A}$ and CK5/6 biosynthesis are controlled conversely.

6.3.3. $sLe^{X/A}$ inhibition leads to increased CK5/6 expression in a breast cancer cell line

To further understand the correlation between $sLe^{X/A}$ and CK5/6, we then analysed a BC cell line known to express high levels of $sLe^{X/A}$/E-SL, the CF1_T [28] [28]. After submitting the cells to 2-FF, an inhibitor of fucosylation, $sLe^{X/A}$ expression was reduced compared to untreated cells, as expected (Figure 6.3A). This is consistent with previous studies where submitting the cells to the same inhibitor concentration (1mM) abrogated the cell adhesion to E-selectin due to the absence of its ligands (Supplementary Figure 6.2). In addition, cytokeratin expression increased 8-fold after treatment (Figure 6.3B). While CK5/6 was associated with poor cancer prognosis by some studies [33], [34], others have shown that CK5/6 downregulation is associated with increased malignancy of cancer cells [35].

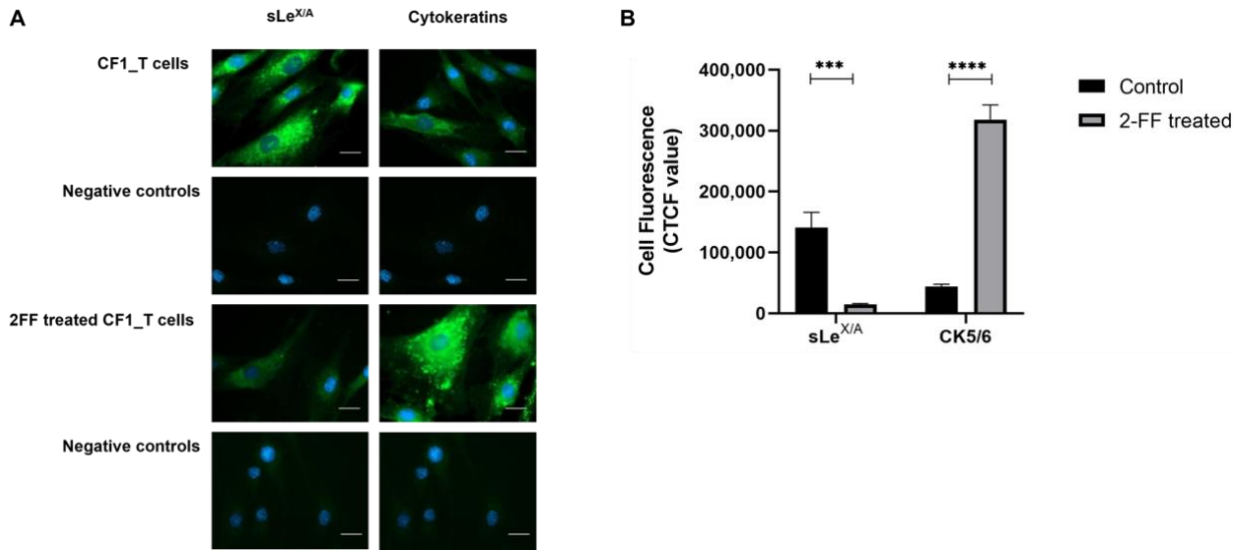

Figure 6.3. Inhibition of fucosylation decreases sLe$^{X/A}$ expression and increases cytokeratin. CF1_T cells were treated or not with 2-fluorofucose (2-FF) and then labelled with HECA-452 mAb and anti-CK monoclonal antibody (mAb), as described in the materials and methods. (A) Fluorescence microscopy images (scale bar: 1 μm). The resulting fluorescence of labelling with HECA-452 and anti-CK is shown per column. The first row presents images of untreated cells stained with primary antibodies; on the second row there are the labelling controls in the absence of primary antibodies; the third row shows CF1_T cells treated with 2-FF and stained with primary antibody, and the fourth row exhibits labelling controls of 2-FF treated cells in the absence of primary antibodies. Images are representative of merging fluorescence where antibody labelling is shown in green, and nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). (B) Corrected total cell fluorescence. The graph shows the calculated arbitrary values of corrected total cell fluorescence (CTCF) retrieved from images depicted in A as described in the materials and methods section. Legend: * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$.

To assess the malignant profile of 2-FF treated cells, which showed increased CK5/6 expression, we set out to study proliferation and invasion in 2-FF treated CF1-T cells. Concordantly with previous reports, the CF1-T cells showed reduced proliferation capacity (Supplementary Figure 6.3) and migratory ability (Supplementary Figure 6.4).

These findings point to a potential mechanism in which sLe$^{X/A}$ and CK5/6 expressions are inversely regulated. It also suggests that patients with lower expression of sLe$^{X/A}$ have reduced TNBC malignant features.

6.3.4. sLe$^{X/A}$ decorates α6 integrin and affects the associated signalling pathways

Since sLe$^{X/A}$ glycan can decorate different cell surface proteins, we considered that the crosstalk between sLe$^{X/A}$ and cytokeratin could be attributed to changes in intracellular signalling coordinated by a sLe$^{X/A}$-decorated protein. This is consistent with our previous observation that the cellular signal transduction pathways are affected upon inhibition of the sLe$^{X/A}$ biosynthesis [28]. To investigate this, we first interrogated which signalling pathways are affected when cells have lower sLe$^{X/A}$ levels due to 2-FF treatment.

The ratio of phosphorylated Src, AKT, ERK1/2 and p38 versus corresponding total protein was assessed by flow cytometry upon 5 days of 2-FF treatment. As shown in Figure 6.4A, the ratio of the expression of phospho-Src/Src and phospho-AKT/AKT showed a significant reduction in 2-FF treated cells. This suggests that Src and AKT pathways are negatively affected when sLe$^{X/A}$ levels are reduced.

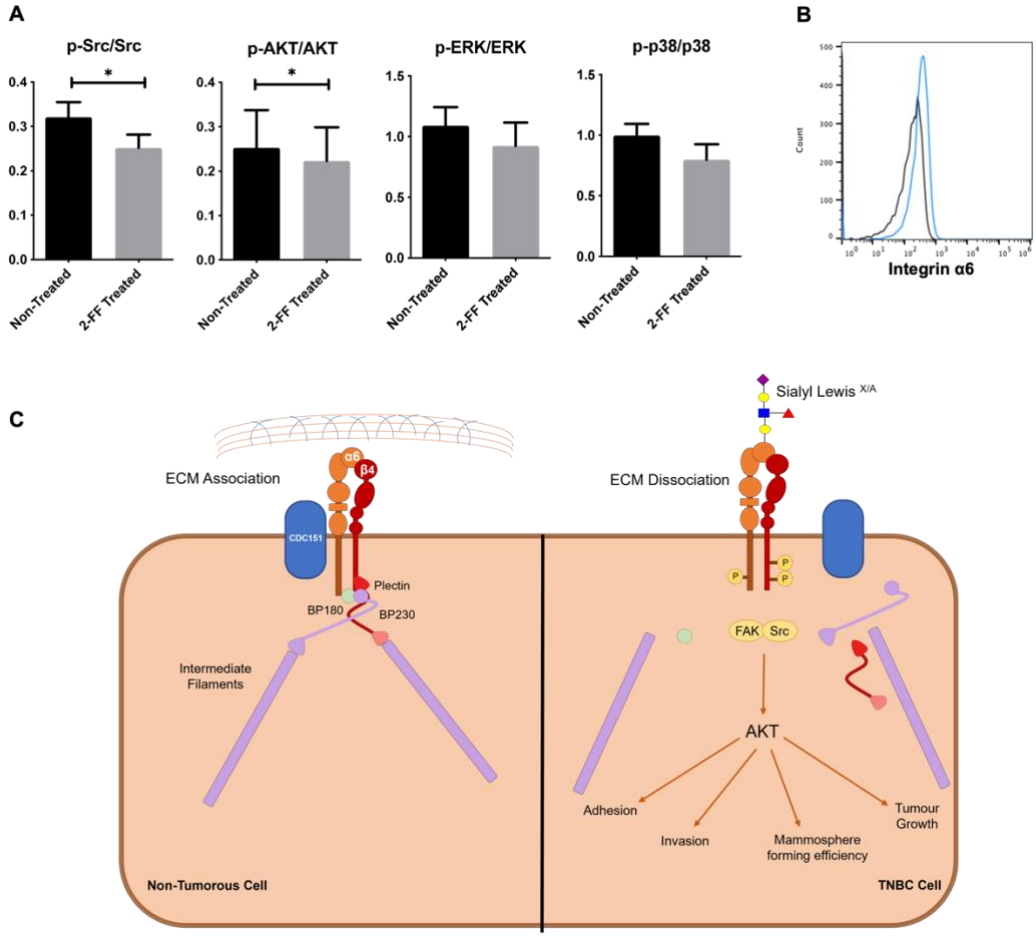

Figure 6.4. α6 integrin-associated pathways are affected upon 2-FF treatment. (A) Expression of phosphorylated proteins is affected by 2-FF. CF1_T cell line treated with 2-FF for 5 days were analysed regarding their expression of phosphorylated Src (p-Src), AKT (p-AKT), ERK1/2 (p-ERK) and respective total proteins by flow cytometry. Ratio between phosphorylated versus total protein expression is represented in the graphs (n= 3, $p < 0.05$ (*)). (B) CF1_T cells express α6 integrin. Flow cytometry analysis of CF1_T cells stained with anti-α6 integrin antibody, plus fluorescent secondary antibody. Representative histogram showing positive staining (blue line). Cells stained only with fluorescent secondary antibody and without anti-α6 integrin antibody were used as negative control (dark line); (C) Proposed mechanism of the influence of sLe$^{X/A}$ decoration of α6integrin on cytokeratin expression. The crosstalk between α6 integrin and cytokeratins is established by the hemidesmosome (complex of α6β4 integrin, plectin, BP180, CD151, BP230). sLe$^{X/A}$ decoration activates integrin and the downstream signalling pathways, contributing to a more aggressive phenotype.

Additionally, we revised which glycoproteins are decorated with sLe$^{X/A}$ (previously reported [20]). Among them, the α6 integrin was identified as a potential player in the link between sLe$^{X/A}$ and cytokeratin with the highest association combined score (Supplementary Table 6.2). In addition, the above identified affected Src and AKT pathways are known to be intermediated by α6 integrin [36]. Since the role of α6 integrin is well established in the

formation of the HD, particularly complexed with β4 integrin [37], we hypothesise that the signalling mechanisms necessary for the good functioning of the HD might be also impaired.

Flow cytometric analysis confirmed the expression of α6 integrin on the cell surface of CF1-T cells (Figure 6.4B). We also confirmed the expression of plectin by these cells, a protein reported to be virtually expressed in all mammalian cells and tissues (Supplementary Figure 6.5). This agrees with the fact that plectin links the cytoplasmic tail of integrin subunit β4 (complexed to α6 integrin) to cytokeratins. Overall, these data further corroborate that the decoration of α6 integrin with $sLe^{X/A}$ contributes to the deregulation of signalling pathways in the interplay between integrin and cytokeratins linked by plectin (Figure 6.4C).

6.4. Discussion

Among the subtypes of BC, TNBC is well known for its heterogeneity and aggressiveness, as well as for its patients having the highest mortality rates. According to our findings, TNBC patients had a DFS of 2-3 years, which is consistent with previous reports of a typical poor prognosis [4], [38]. The highly variable response between each patient with TNBC prevents patients from being uniformly treated, urging the need for a better understanding of the underlying mechanism for better subtyping that helps prognosis and selection of appropriate therapy for these patients.

In cancer, $sLe^{X/A}$ antigens play an important role in cell migration and metastasis by facilitating extravasation acting as selectin ligands, and participating in tumorigenic signalling [20], [28], [39]–[41]. Despite its well-known association with metastatic lesions in BC [17], [25], its significance in TNBC is still unknown. Therefore, to the best of our knowledge, there is a need to further investigate $sLe^{X/A}$ potential role in TNBC metastasis. In the current study, all tested TNBC samples were stained for $sLe^{X/A}$ and E-SL in addition to at least one basal marker, CK5/6, EGFR, AR or P-cadherin, as previously described [42], [43]. Our data showed a strong correlation between $sLe^{X/A}$ and E-SL reactivity suggesting that $sLe^{X/A}$ glycans are the major ligands of E-selectin in TNBC [27].

When comparing the $sLe^{X/A}$ results to the other markers studied, we encountered a negative correlation with CK5/6 which is an important intermediate filament involved in cell adhesion and migration [44]. Interestingly, higher $sLe^{X/A}$ expression and lower CK5/6 expression led to a faster disease recurrence when looking at their DFS. *In silico* analysis of the gene expression of CK5/6 regarding the gene expression of the α1,3-fucosyltransferases that catalyse the terminal fucosylation steps (FUT3, FUT4, FUT5, FUT6, FUT7, and FUT10) (Figure 2D) [18], [21] revealed a negative association of *FUT6* and *FUT10* with the CK5/6 genes. These findings help to further corroborate a possible antagonistic interaction between the $sLe^{X/A}$ biosynthetic pathway and the CK5/6 pathway.

Since $sLe^{X/A}$ protein scaffolds are critical for cell signalling, it is likely that these proteins strongly influence the CK5/6 expression. Our group previously reported which glycoproteins

are decorated with $sLe^{X/A}$ in BC [20]. Using the STRING database [45] of reported protein-protein interactions and computational prediction, we identified that α6 integrin has the highest interaction score with CK5/6 protein. In cancer, α6 integrin is often associated with metastatic behaviour, increasing cancer cells' migration and invasion, due to the disassembly of the HD [46] [46]. Concordantly, other studies also report that altered glycosylation promotes the phosphorylation of the subunit β4 of integrin and metastasis [47].

To understand the distinctions between the presence and absence of $sLe^{X/A}$ CF1_T cell line was treated with 2-FF, a small molecule fucosyltransferase inhibitor, which inhibits $sLe^{X/A}$ expression. Upon treatment, an increase of CK5/6 expression was observed which further supports prior data regarding a potential opposing interplay between CK5/6 and $sLe^{X/A}$. Treated CF1_T cells also displayed a reduced cancer cell migration and proliferation, which is consistent with the $sLe^{X/A}$ association with a lower DFS. We identified differences between treated and untreated cell lines suggesting the potential involvement of the Src pathway which is a major player in BC cell proliferation and migration [48]. Src acts as a signalling cue for other pathways such as AKT, which was also altered after 2-FF treatment, and EGFR, which was expressed in 70% of the examined patients' tissue. This information, together with the observation that α6 integrin has the highest CK5/6 interaction score, might indicate that $sLe^{X/A}$ expression in α6 integrin activates the Src pathway. The activation of this pathway could result in: i) a decrease of the cytokeratin, as cancer cells undergo epithelial-to-mesenchymal transition necessary for a metastatic profile; and ii) phosphorylation of α6β4 integrin, further facilitated by the decrease of cytokeratin to promote the dissociation of HD. Altogether, these interactions contribute to the migratory and more malignant behaviour of higher expression of $sLe^{X/A}$ TNBC cells.

We recognize some limitations in this study that could be addressed in future research. First, given that this study was achieved through a direct collaboration with the Breast unit of the CUF Oncology Institute (which works exclusively with patients diagnosed with BC), it was not possible to obtain normal samples from apparently healthy individuals. Future studies should include normal samples or, alternatively, peritumoral tissue from the TNBC cancer patients as controls. Second, efforts should be made to validate our observations in a bigger patient cohort. Not only this can increase the strength of the observed correlation but also might allow patient stratification according to $sLe^{X/A}$ levels or other biomarkers. Nevertheless, our analysis was complemented with gene expression data of 160 cases of TNBC retrieved from the TCGA database which reinforced in part the limitation of our patient cohort. It is also important to discuss that we used the CF1_T cell line as one of three models in our study. This cell line, originally obtained from a patient with invasive ductal carcinoma, was selected as a model due to its very low expression of CK5/6 and high expression of $sLe^{X/A}$ [28]. The expression of ER, PR and HER2 biomarkers is also low. When treated with 2-FF, concurrently with a reduction in $sLe^{X/A}$, there is an increase in CK5/6 expression analysed by microscopic fluorescence, similarly to what is seen when there is low $sLe^{X/A}$ expression in TNBC tissues. The

use of this cell line to complement the information derived from TNBC tissues and described in the TCGA dataset supports the inverse crosstalk between $sLe^{X/A}$ and CK5/6 and suggests that our findings may be transversal to other BC types. Finally, other models and lines of research could be used or developed to continue deciphering the reported associations and proposed pathway. Therefore, besides that our results must be confirmed in other *in vitro/in vivo* TNBC models and in a bigger TNBC patient cohort, this study opens new research avenues to study these mechanisms in TNBC and other BC types. These findings contribute to highlight malignant features to be explored as potential therapeutic targets.

6.5. Conclusions

Our findings contribute to new understanding of TNBC cells adhesion and metastatic behaviour, highlighting a previously unknown crosstalk between $sLe^{X/A}$ and cytokeratin, intermediated by the α6 integrin. Further studies are necessary to clarify the interaction and the role of the proteins involved in this process. Our contribution supports $sLe^{X/A}$ as a critical player in TNBC malignancy and suggests it as a biomarker to target and treat this BC type.

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CHAPTER 7.

DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

Glycosylation is an essential biological process with implications on the structure and function of proteins and other molecules, consequently with a role in virtually all bodily functions. Just as glycosylation is key for the normal operation of the organism, the disruption or shift in its action has extreme consequences that lead to complex, debilitating, and life-threatening diseases [1].

People affected by chronic, rare, or infrequent diseases have unique and burdensome clinical presentations, which often require a long period of treatment and complex medical care and hinder their QoL [2], [3]. Besides, diseases affecting small populations are normally and scientifically misunderstood, due to small and dispersed cohorts, lack of models and even lack of awareness among doctors and researchers [4]. This scenario requires the medical and scientific community to adapt in order to equitably answer immediate needs and leaving no patient behind. With this new paradigm, patient-centricity emerges as a solution to not only identify patients' needs, but also to guarantee the relevance, adequacy, and meaningfulness of research [5].

The overarching goal of this thesis was to apply a patient-centric methodology to answer community-identified needs of two glycosylation-related diseases, namely PMM2-CDG and TNBC, particularly focusing on their immunological features.

Chapter 3 specifically aimed to identify, characterise, and validate fibroblasts as a cellular model for the study of immunological mechanisms to help mitigate the lack of (or access to) samples and appropriate disease models, a need identified in CDG research [6]. Results show that patient-derived fibroblasts respond to inflammatory stimulus by activating similar signalling pathways to those of immune cells, which provides an alternative method to study these pathways when other models are unavailable. In fact, specifically for PMM2-CDG, several animal disease models have been developed or attempted, but they fail to resemble the complete multi-system patient phenotypes [7]–[9]. Most often, homozygous *PMM2* knockout mice models were associated with incompatibility with life [10]. Therefore, the learnings from this study, along with efforts from other teams [11], [12], validating cellular models that reciprocate patient-derived cells for immunological studies are a significant contribution to a

fundamental need. Nonetheless, validation using bigger patient cohorts and the development of models that represent the genetic and phenotypical variability in PMM2-CDG requires further attention. This study, however, provides an integrative platform transposable to any genetic RD that can aid research efforts in their pathology as well as to provide diagnosis and treatment clues.

After establishing patient-derived fibroblasts as an appropriate platform and characterizing their inflammatory response, we used them as a cellular model to investigate the impact of abnormal glycosylation in the immune function of PMM2-CDG patients, a highly clinically impactful and neglected research topic. The results of our comprehensive methodology, described in **Chapter 4**, identified faulty inflammatory molecular mechanisms and dysregulated players in severe pathogenic variants of PMM2-CDG. Particularly, under an inflammatory challenge (TNF-α stimulation), PMM2-CDG cells were found to have surface glycosylation defects that seem to affect the TNFR1 both structurally and functionally. First, abnormal TNFR1 shedding was observed in PMM2-CDG along with dysregulated signal transduction players, translating in defective ERK1/2, P38 and JNK signalling and immune effectors (i.e., IL-6 and CCL5) - important for an adequate inflammatory response [13]–[17]. These findings are in line and explain, at least in part, the severe and difficult to resolve infections that afflict PMM2-CDG patients, hinting towards potential immunotherapeutic targets. Besides answering its specific aim of unravelling mechanisms of the immunopathology of PMM2-CDG, this chapter raised follow-up questions and research avenues:

- How exactly the PMM2-CDG-related glycosylation abnormalities affect TNFR1 downstream signalling still needs to be investigated, particularly, if the structure of the receptor is affected by increased activity of TACE due to its glycosylation status or if ligand-receptor affinity can be impaired [18]–[20].
- The identification of muscle-related alterations and possible link with fibrosis during inflammation, highlights the possible interconnection between the immune and other systems. This is in line with the previous observation correlating gastrointestinal involvement with the presence of infections in PMM2-CDG [21]. Besides, the presence of infections and subsequent inflammatory processes can further trigger glycosylation shifts that globally aggravate other body tissues or organs [22]. Therefore, further research is needed into the interconnection of the multiple system affected in PMM2-CDG.

There are also other important factors to consider when investigating the immune involvement of PMM2-CDG. First, it is possible that the immunophenotype of PMM2-CDG is influenced by a dual-effect of glycosylation deficits. It can not only be a consequence of impaired immunological responses, as shown by us herein, but also, the increased frequency of infections might be due to an augmented capacity of microorganisms to infect the host with more favourable glycosylation patterns. In fact, it was previously shown that PMM2-CDG patients have increased expression of glycosphingolipids, particularly, globotriaosylceramide

[23], which is not only a toxin receptor, but also a binding site for several bacterial adhesins [24]–[26]. Besides, there is a predominance of short mannose-terminated glycans in PMM2-CDG [27], [28], which have roles in the adhesion and infection by several microorganisms [29]. Hypersialylation was also found in PMM2-CDG cells which is known to facilitate some viral infections (e.g., Influenza) [30]. Nevertheless, one should also consider that just as the entrance of some pathogens can be favoured by the shift towards specific glycan motifs, in other cases, the glycosylation modifications observed might also confer resistance to other infectious agents, like in the case MOGS-CDG and ATP6AP1-CDG [31], [32].

The infection frequency and severity, their debilitating consequences as well as remaining multi-factorial burden of PMM2-CDG result in impaired QoL [21], [33]. However, there are no disease-specific tools to quantify the QoL impairment, its variation across time, phenotypes, or therapeutic interventions. In an era where patient-centred outcomes are escalating with increasingly important roles in therapy evaluation and approval, identifying or developing appropriate tools that consider the PMM2-CDG specificities is essential. **Chapter 5** aimed to answer this need by the application of a community-guided methodology. The perspectives of the patient and professional community were collected to not only identify the real-world most impactful signs and symptoms in patients' daily lives, but also to complement this information with clinical relevance. This allowed a targeted revision and analysis of HrQoL PRO specific for those impactful manifestations, ensuring their significance, which is a step towards the development of a PMM2-CDG-specific HrQoL measure, contributing towards the therapy evaluation and approval phase of R&D. While this study successfully applied an innovative methodology and identified a plethora of adaptable instruments for PMM2-CDG-related conditions, several barriers to their application exist due to the nature of the disease. Specifically, neurological impairments raise the need for proxy- rather than self-reporting which provide approximate measurements of QoL [34]. Besides, the clinical variability between patients and across one patient lifetime difficult the prospect for a sole and static HrQoL tool. For such complex diseases, the creation of a patient/caregiver-friendly modular questionnaire constitute an attractive initiative for which the identification of symptom-specific tools derived from this work can greatly contribute to [35].

Chapter 6 focuses on the efforts developed towards the clarification of TNBC heterogeneity which is an urgent gap impeding the development of targeted treatments and, consequently, impairs patients' QoL, as reported by the community. To help answer this need, this chapter demonstrated the studies undertaken to unveil the clinical and functional role of the glycan-based tumour associated antigen $sLe^{X/A}$ in TNBC for the first time. Clinically, tumours expressing high levels of $sLe^{X/A}$ experienced less DFS. Biologically, $sLe^{X/A}$ expression associated with an increased proliferation and invasion capacity. This finding highlights $sLe^{X/A}$ as a contributing factor for the metastatic behaviour of TNBC, like seen in other BC types [36], [37], and identifies its potential for TNBC stratification, as a prognosis biomarker and as a therapeutic target. Interestingly, a previously unreported mechanistic negative correlation

between $sLe^{X/A}$ and CK5/6 was observed which, along with disrupted signalling, allowed to suggest the $sLe^{X/A}$-carrier protein α6 integrin as a mediator. Indeed, given the known roles of this integrin in the HD complex and, therefore, cellular adhesion to the basement membrane, it is possible that $sLe^{X/A}$ integrin decoration impairs this mechanism [38]. The fact that this process was shown to be affected in other types of cancer and associated with cancer aggressiveness reinforces our hypothesis [39], [40]. Nevertheless, further studies need to be undertaken to further explore the exact consequences of the activation of Src and AKT signalling by $sLe^{X/A}$. Specifically, it is possible that cancer cells undergo epithelial-to-mesenchymal transition necessary for a metastatic profile due to a direct decrease in CK5/6 expression like what happens with other cytokeratins in BC [41]. Alternatively, a consequent phosphorylation of α6β4 integrin might lead to the dissociation of the HD promoting the migratory behaviour [42].

The application of a people-centric framework to research is illustrated in this thesis in several cases from the focus in public-selected topics and identified needs as well as by actively involving the community with advisory roles, orienting, and revising research. Nevertheless, strategies to adopt patient-centric and PI strategies should be tailored and adopted according to the target population, hypothesis, goals, and expectations. The continued demonstrated benefits of patient-centricity and PI to people, researchers, and the society will hopefully continue to unfold and attract researchers to adopt this mindset. Consequently, people can increasingly feel more included, knowledgeable, and empowered increasingly adding value to the cycle.

In conclusion, this thesis stresses the impact of glycosylation alterations in PMM2-CDG and TNBC, particularly in what concerns their immunological features, and highlights related targets that can aid stratification and treatment development. Besides, it illustrates the benefits of adopting patient-centric approaches and patient-oriented methods that can translate in better research and future clinical outcomes.

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A.

SUPPLEMENTARY MATERIAL

CHAPTER 2.

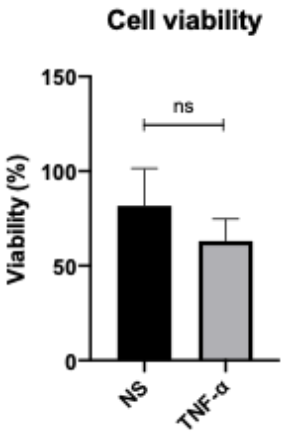
SUPPLEMENTARY MATERIAL

Supplementary File 2.1. CDG classification table (.xlsx). The table shows the 163 CDG-related genes their associated 217 different diseases or phenotypes, classified according to the affected glycosylation pathways, namely N-glycosylation, O-glycosylation, GPI-anchor synthesis, lipid glycosylation, and multiple and other glycosylation pathways. The table also depicts the inheritance pattern, affected organs and systems, the biochemical pathway and cellular location, the TIEF pattern, biomarkers, disease models, targeted treatment, and the year of first reported/genetically confirmed patient of each disease. Available at https://bit.ly/CDGClassificationTable.

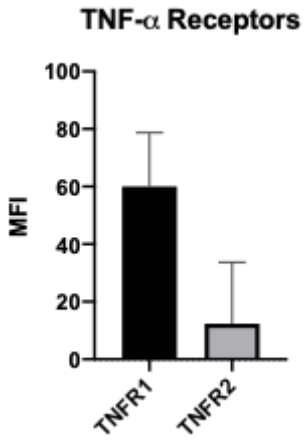
CHAPTER 3.

SUPPLEMENTARY MATERIAL

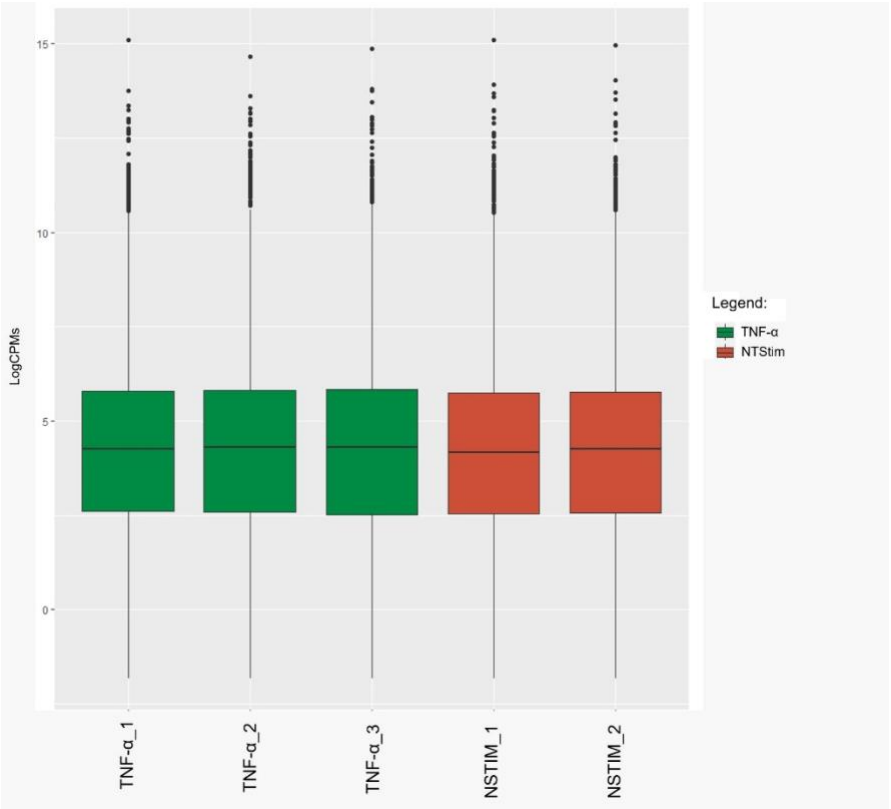
Supplementary File 3.1. Cell-cell predicted interactions based on ligand/receptor gene expressions (.xlsx). List of putative receptor-ligand pairs based on the genes that are differentially expressed upon TNF-α stimulation (DEG) obtained from the Supplementary Data 2 of a recent article related to the FANTOM 5 project (44). Each pair has its ligand and receptor described as well as which one is differentially expressed in the stimulated fibroblasts (DEGs_fibroblasts column) or the possible interacting cells (Immune cells column). Finally, we assessed the gene expression of the receptor/ligands of immune cells (monocytes, B cells, T cells, DCs, eosinophils, macrophages, NK cells and neutrophils) based on the Immune cells expression sheet. Specifically, "1" stands for detectable expression in the immune cell line. The Immune cells expression sheet contains the gene expression (transcripts per million) of receptors and ligands possibly present in immune cells of the receptor-ligand pairs described in the Fibroblasts_immune interactions sheet, obtained from the Supplementary Data 4 of a recent article related to the FANTOM 5 project (44). According to previous reports, we considered a cell type to express a certain gene if they have more than 10 transcripts per million (highlighted in green). Some genes were not detected which we highlighted in red. Available at https://bit.ly/CellCellPredictedInteractions.



Supplementary Figure 3.1. Cell viability upon TNF-α stimulation. Cells were stimulated or not with 10 ng/ml of TNF-α for 24 h and cells were counted using the automatic cell counter. Data are represented as the mean ± SD (n = 3). There is no significant difference between the cell viability between condition bases on an unpaired t-test. Ns – not significant



Supplementary Figure 3.2. Cell surface expression of the TNF-α receptors of the fibroblasts under study. Fibroblasts were stimulated or not with 10 ng/ml of TNF-α for 24 h and the receptors analysed by flow cytometry using the PE- conjugated anti-human CD120A or the APC-conjugated CD120B (Biolegend). Data are represented as the mean ± SD (n = 3). MFI – Median fluorescence intensity; TNFR – Tumour necrosis factor receptor.



Supplementary Figure 3.3. Boxplots of log(CPM) values showing the gene expression distributions of all samples. The line dividing the boxes represents the median of the gene expression and the top and bottom of the box shows the upper and lower quartiles, respectively. The whiskers show the highest and lowest gene expression values. Outliers are shown as circles. NSTIM = Non-stimulated

Supplementary File 3.2. Differential expressed genes (DEG) between TNF-α stimulated and non-stimulated fibroblasts (.xlsx). DEG were obtained using the "edgeR'' package in the R (v 4.1.1) using the quality controlled and filtered read counts and a False Discovery Rate (FDR) ≤ 0.05 as cut-off. Upregulated and downregulated DEG are separated in different sheets. Available at https://bit.ly/DEGFibroblasts.

Supplementary File 3.3. Gene set enrichment analysis (GSEA) of TNF-α stimulated fibroblasts (.xlsx). Enriched targets of transcription factors, Reactome-pathways, and gene ontology (GO) biological processes obtained from a GSEA, carried out with the WEB-based GEne SeT AnaLysis Toolkit 2019 [1] FDR ≤ 0.05 and a minimum of 5 and maximum 2000 genes per category were set as the cut-off criteria (.xlsx). Available at https://bit.ly/GSEAFibroblasts.

Supplementary Table 3.1. Enriched transcription factors' targets upon human skin fibroblasts TNF-α stimulation. Significant transcription factors' targets were identified by a gene set enrichment analysis and the associated motifs were retrieved from the molecular signatures database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp).

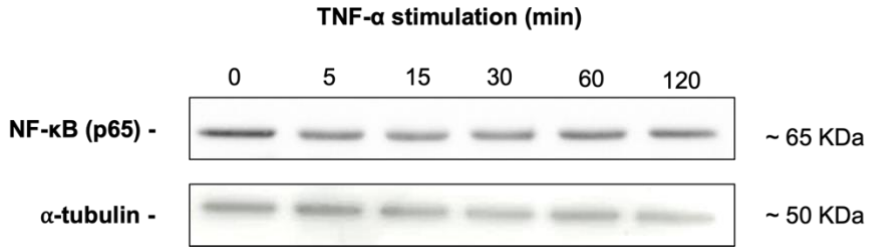
geneSet	Motif ID	Motif	NES	FDR
V$NFKAPPAB_01	M1	GGGAMTTYCC	3,6855	0
V$NFKB_Q6_01	M2	NNNNKGGRAANTCCCN	3,6681	0
V$NFKB_C	M3	NGGGACTTTCCA	3,4558	0
V$NFKAPPAB65_01	M4	GGGRATTTCC	3,3479	0
V$NFKB_Q6	M5	NGGGGAMTTTCCNN	3,3394	0
GGGNNTTTCC_V$NFKB_Q6_01	M6	GGGNNTTTCC	3,3292	0
V$CREL_01	M7	SGGRNTTTCC	3,2078	0
V$ISRE_01	M8	CAGTTTCWCTTTYCC	2,6716	0,0001
STTTCRNTTT_V$IRF_Q6	M9	STTTCRNTTT	2,6593	0,0001
V$STAT5B_01	M10	NAWTTCYNGGAAWTN	2,4893	0,0006
V$IRF7_01	M11	TNSGAAWNCGAAANTNNN	2,3492	0,0025
TTANTCA_UNKNOWN	M12	TTANTCA	2,2785	0,0038
V$STAT5A_01	M13	NAWTTCYNGGAANYN	2,2330	0,0062
V$RP58_01	M14	NNAACATCTGGA	2,1753	0,0086
TTCYNRGAA_V$STAT5B_01	M15	TTCYNRGAA	2,1889	0,0089
V$NFAT_Q4_01	M16	NWGGAAANWN	2,1761	0,0091
V$ICSBP_Q6	M17	RAARTGAAACTG	2,1412	0,0118
TGGNNNNNNKCCAR_UNKNOWN	M18	TGGNNNNNNKCCAR	2,1245	0,0133
V$STAT3_01	M19	NGNNATTTCCSGGAARTGNNN	2,0997	0,0146
V$CEBPA_01	M20	NNATTRCNNAANNN	2,1022	0,0149
V$IRF_Q6	M21	BNCRSTTTCANTTYY	2,0846	0,0154
V$ELF1_Q6	M22	RNWMBAGGAART	2,0721	0,0168
V$STAT1_03	M23	NNTTTCCN	2,0573	0,0184
RGAANNTTC_V$HSF1_01	M24	RGAANNTTC	2,0460	0,0189
TTGCWCAAY_V$CEBPB_02	M25	TTGCWCAAY	2,0191	0,0231
V$IRF2_01	M26	GAAAAGYGAAASY	2,0092	0,0246
V$CEBPDELTA_Q6	M27	MATTKCNTMAYY	1,9811	0,0304
V$FREAC4_01	M28	CTWAWGTAAACANWGN	1,9289	0,0438
V$STAT6_02	M29	NNYTTCCY	1,9301	0,0447
V$HIF1_Q5	M30	CGTACGTGCNGB	1,9319	0,0458
V$LEF1_Q6	M31	SWWCAAAGGG	1,9196	0,0460

Legend: NES - normalised enrichment score; FDR - false discovery rate.

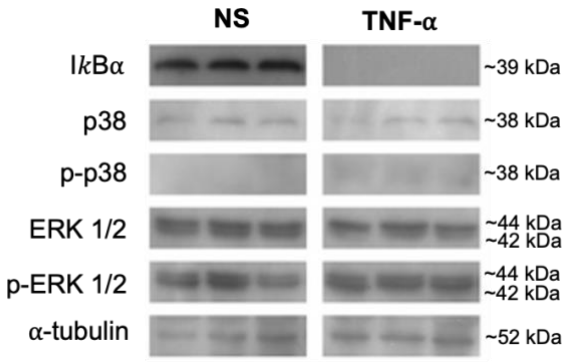
Supplementary Table 3.2. Cytokine and chemokine production of non-stimulated (NStim) and TNF-α stimulated samples. Individual measurements of cytokines and chemokines concentration of NStim and TNF-α stimulated samples (10 ng/ml, t = 24 h) in the three fibroblast lines under study. Cytokines (IL-1β, IL-4, IL-6, IL-10, IL-12, IL-15 and IFN-γ) were measured by ELISA while chemokines (CXCL8, CCL2, CCL5, CXCL1 and CXCL5) levels were analysed using a Mix and Match LegendPlex kit, as described in Materials and Methods. Data represent normalized values obtained by dividing the concentration of the cytokine/chemokine (pg/ml) by the concentration of total protein (mg/ml); values represent the mean of 3 technical replicates.

	NStim			*TNF-a*		
Cytokine	NStim 1	NStim 2	NStim 3	TNF-α 1	TNF-α 2	TNF-α 3
IL-1ß	0	0	0	0	0	0
IL-4	0	0	0	0	0	0
IL-6	0.29	0.26	0.26	4.6	12.47	11
IL-10	0.1	0.1	0.26	0.02	0	0
IL-12	0	0.17	0.06	0	0	0
IL-15	0	0	0	0	0	0.1
IFN-γ	0	0	0	0	0	0
CXCL8	0	0.1	0.1	28.6	93.8	83
CCL2	0.2	0.3	0.2	18.2	59.7	52.8
CCL5	0	0	0	0.1	0.2	0.1
CXCL5	0	0	0	0.2	1.4	0.8
CXCL1	0	0.1	0	1.8	9.1	6.3

Legend: NStim – Non-stimulated



Supplementary Figure 3.4. Immunodetection of the expression of the p65 subunit of NF-κB by Western blot. Fibroblasts were stimulated with TNF-α from 0 to 120 min and proteins separated and transferred as described in Material and Methods. Anti-NF-κB-p65 was used followed by an anti-mouse IgG-HRP and detected with SuperSignal® West Femto Maximum Sensivity Substrate (Thermo Fisher Scientific) in a ChemiDoc™ MP imaging system from Bio-Rad Laboratories. After membrane stripping, α-tubulin was detected as a loading control.

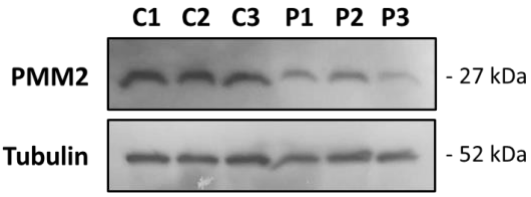


Supplementary Figure 3.5. Immunodetection of the expression of cell signalling proteins by Western blot. Fibroblasts were stimulated with TNF-α for 30 min and proteins separated and transferred as described in Material and Methods. Anti-ERK1/2, anti-p-ER1/2, anti-p38 and anti-p-p38 were used followed by the peroxidase AffiniPure donkey anti-rabbit IgG (H+L) and detected with Lumi-Light Western Blotting Substrate (Roche) to X-ray films (Amersham HyperfilmTM ECL). After membrane stripping, α-tubulin was detected as a loading control and normalization protein.

CHAPTER 4.

SUPPLEMENTARY MATERIAL

Supplementary File 4.1. Cell-cell predicted interactions based the DEG of WT and PMM2-CDG TNF-α stimulated fibroblasts and on ligand/receptor gene expressions (.xlsx). List of putative receptor-ligand pairs based on the genes that are differentially expressed upon TNF-α stimulation (DEG) of both WT and PMM2-CDG TNF-α stimulated fibroblasts obtained from the Supplementary Data 2 of a recent article related to the FANTOM 5 project (44). Each pair has its ligand and receptor described as well as which one is differentially expressed in the stimulated fibroblasts (DEGs_fibroblasts column) or the possible interacting cells (Immune cells column). Finally, we assessed the gene expression of the receptor/ligands of immune cells (monocytes, B cells, T cells, DCs, eosinophils, macrophages, NK cells and neutrophils) based on the Immune cells expression sheet. Specifically, "1" stands for detectable expression in the immune cell line. The Immune cells expression sheet contains the gene expression (transcripts per million) of receptors and ligands possibly present in immune cells of the receptor-ligand pairs described in the Fibroblasts_immune interactions sheet, obtained from the Supplementary Data 4 of a recent article related to the FANTOM 5 project (44). According to previous reports, we considered a cell type to express a certain gene if they have more than 10 transcripts per million (highlighted in green). Some genes were not detected which we highlighted in red. Available at https://bit.ly/CellCellInteractionsWTPMM2.



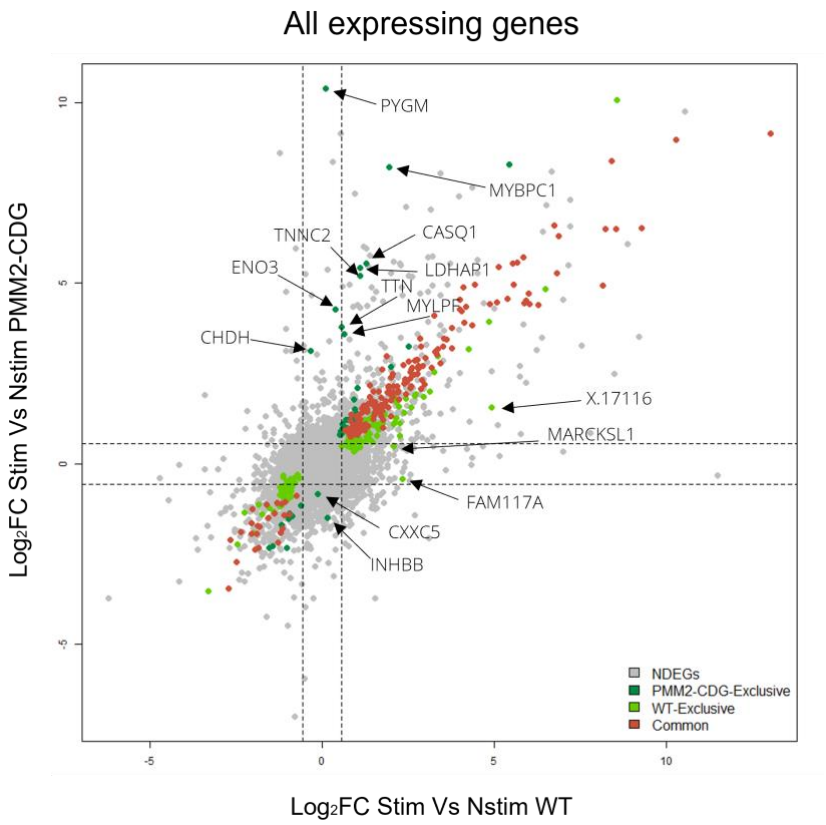
Supplementary Figure 4.1. Western blot analysis of the PMM2 protein in PMM2-CDG and WT skin fibroblasts. Immunoblotting of cell lysates from 3 PMM2-CDG and WT fibroblasts was performed using anti-PMM2 (Proteintech) at 1:1000. Mouse monoclonal anti-α-tubulin staining was performed as loading control.

Supplementary File 4.2. Differential expressed genes of WT and PMM2-CDG skin fibroblasts (.xlsx). The differentially expressed genes (DEG) between TNF-α stimulated and non-stimulated PMM2-CDG and WT fibroblasts were obtained using the "edgeR" (v 3.36) package in with a cut-off of False Discovery Rate (FDR) ≤ 0.05). DEG were divided in 6 different categories: (1) upregulated DEGs common between WT and PMM2-CDG samples (CommonDEG_Pos); (2) downregulated DEGs common between WT and PMM2-CDG samples (CommonDEG_Neg) positive; (3) upregulated WT-exclusive DEG (WT_exclusive_pos); (4) downregulated WT-exclusive DEG (WT_exclusive_neg); (5) upregulated PMM2-exclusive DEG (PMM2_exclusive_pos) and (6) downregulated PMM2-exclusive DEG (PMM2_exclusive_neg). The list of all expressed genes (detected transcripts) is included (Background genes). Available at https://bit.ly/DEG_WT_PMM2.

Supplementary File 4.3. Enriched gene ontology (GO) terms common between WT and PMM2-CDG stimulated fibroblasts (.xlsx). The GO terms (particularly biological processes, molecular functions, and cellular components) enriched in TNF-α stimulated WT and PMM2-CDG fibroblasts were obtained following an over-representation analysis biological processes using the Toppfun functionality of the Toppgene Suite (v 31) platform [33]. FDR adjusted p ≤ 0.05 was set as the cut-off criteria. Common GO terms between WT and PMM2-CDG TNF-α stimulated fibroblasts are depicted and categorized according to if they were obtained from the upregulated (up) or downregulated (down). Available at https://bit.ly/CommonGOTerms.

Supplementary File 4.4. Enriched WT-exclusive gene ontology (GO) terms (.xlsx). The GO terms (particularly biological processes, molecular functions, and cellular components) enriched in TNF-α stimulated WT and PMM2-CDG fibroblasts were obtained following an over-representation analysis biological processes using the Toppfun functionality of the Toppgene Suite (v 31) platform [33]. FDR adjusted p ≤ 0.05 was set as the cut-off criteria. GO terms enriched exclusively in TNF-α stimulated WT samples are depicted and categorized according to if they were obtained from the upregulated (up) or downregulated (down). Available at https://bit.ly/WTExclusiveGOterms.

Supplementary File 4.5. Enriched PMM2-exclusive gene ontology (GO) terms (.xlsx). The GO terms (particularly biological processes, molecular functions, and cellular components) enriched in TNF-α stimulated WT and PMM2-CDG fibroblasts were obtained following an over-representation analysis biological processes using the Toppfun functionality of the Toppgene Suite (v 31) platform [33]. FDR adjusted p ≤ 0.05 was set as the cut-off criteria. GO terms enriched exclusively in TNF-α stimulated PMM2-CDG samples are depicted and categorized according to if they were obtained from the upregulated (up) or downregulated (down). Available at https://bit.ly/PMM2ExclusiveGOTerms.



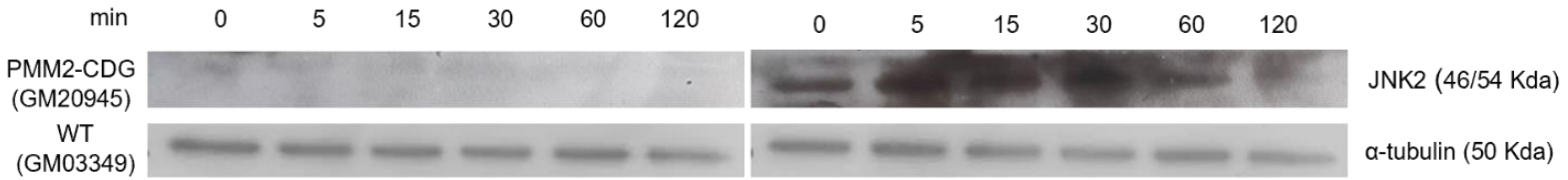
Supplementary Figure 4.2. Expression plot of all expressed genes. This scatter plot illustrates the comparison of expression of the same gene between sample groups upon TNF-α, where the X-axis is the log2FC of stimulated (Stim) WT vs non-stimulated (NStim) WT and the Y-axis shows the log2FC of Stim PMM2-CDG vs NStim PMM2-CDG. The selected lines are placed in the 0.56 of log2FC both in the X- and Y-axis, which is the lowest log2FC in both groups. Grey dots represent all non-DEG; dark green dots comprise all the DEG in the PMM2-CDG-exclusive group; light green dots compose the DEG of the WT-exclusive group and red dots consist of all Common DEG between sample groups. The identified DEG correspond to those which change in expression (logFC) is most different between WT and PMM2-CDG samples upon TNF-α stimulation.

Supplementary Table 4.1. Function and log2FC of DEG with most dissimilar changes in expression levels in response to TNF-α stimulation. DEG with the most contrasting expression levels are highlighted in Figure S2. Protein function was retrieved from the UniProt database. N/A – Not available.

Gene (protein)	Function[a]	Log2FC WT	Log2FC PMM2-CDG
	PMM2-CDG-exclusive DEG		
PYGM **(Glycogen phosphorylase, muscle form)**	Allosteric enzyme that catalyses the rate-limiting step in glycogen catabolism, the phosphorolytic cleavage of glycogen to produce glucose-1-phosphate, and plays a central role in maintaining cellular and organismal glucose homeostasis.	0.09	10.39
MYBPC1 **(Myosin-binding protein C, slow-type)**	Thick filament-associated protein located in the crossbridge region of vertebrate striated muscle a bands. Slow skeletal protein that binds to both myosin and actin. In vitro, binds to native thin filaments and modifies the activity of actin-activated myosin ATPase. May modulate muscle contraction or may play a more structural role	1.95	8.22
CASQ1 **(Calsequestrin-1)**	Calsequestrin is a high-capacity, moderate affinity, calcium-binding protein and thus acts as an internal calcium store in muscle. Calcium ions are bound by clusters of acidic residues at the protein surface, often at the interface between subunits. Can bind around 80 Ca2+ ions. Regulates the release of luminal Ca2+ via the calcium release channel RYR1; this plays an important role in triggering muscle contraction. Negatively regulates store-operated Ca2+ entry (SOCE) activity.	1.27	5.55
LDHAP1 **(Lactate Dehydrogenase A Pseudogene 1)**	NA *(A pseudogene is a segment of DNA that structurally resembles a gene but is not capable of coding for a protein.)*	1.09	5.42
TNNC2 **(Troponin C, skeletal muscle)**	Troponin is the central regulatory protein of striated muscle contraction. Tn consists of three components: Tn-I which is the inhibitor of actomyosin ATPase, Tn-T which contains the binding site for tropomyosin and Tn-C. The binding of calcium to Tn-C abolishes the inhibitory action of Tn on actin filaments.	1.09	5.21
TTN **(Titin)**	Key component in the assembly and functioning of vertebrate striated muscles. By providing connections at the level of individual microfilaments, it contributes to the fine balance of forces between the two halves of the sarcomere. The size and extensibility of the cross-links are the main determinants of sarcomere extensibility properties of muscle. In non-muscle cells, seems	0.59	3.78

	to play a role in chromosome condensation and chromosome segregation during mitosis. Might link the lamina network to chromatin or nuclear actin, or both during interphase.		
MYLPF **(Myosin regulatory light chain 11)**	Myosin regulatory subunit that plays an essential role to maintain muscle integrity during early development (By similarity). Plays a role in muscle contraction (By similarity).	0.65	3.60
CHDH **(Choline dehydrogenase, mitochondrial)**	Enzyme catalysing the dehydrogenation of *choline* to betaine aldehyde in *mitochondria*	-0.35	3.11
ENO3 **(Beta-enolase)**	Glycolytic enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Appears to have a function in striated muscle development and regeneration.	0.37	4.26
CXXC5 **(CXXC-type zinc finger protein 5)**	May indirectly participate in activation of the NF-kappa-B and MAPK pathways. Acts as a mediator of BMP4-mediated modulation of canonical Wnt signalling activity in neural stem cells (By similarity). Required for DNA damage-induced ATM phosphorylation, p53 activation and cell cycle arrest. Involved in myelopoiesis. Transcription factor. Binds to the oxygen responsive element of COX4I2 and represses its transcription under hypoxia conditions (4% oxygen), as well as normoxia conditions (20% oxygen). May repress COX4I2 transactivation induced by CHCHD2 and RBPJ. Binds preferentially to DNA containing cytidine-phosphate-guanosine (CpG) dinucleotides over CpH (H=A, T, and C), hemimethylated-CpG and hemimethylated-hydroxymethyl-CpG.	-0.14	-0.84
INHBB **(Inhibin beta B chain)**	Inhibins and activins inhibit and activate, respectively, the secretion of follitropin by the pituitary gland. Inhibins/activins are involved in regulating a number of diverse functions such as hypothalamic and pituitary hormone secretion, gonadal hormone secretion, germ cell development and maturation, erythroid differentiation, insulin secretion, nerve cell survival, embryonic axial development or bone growth, depending on their subunit composition. Inhibins appear to oppose the functions of activins.	0.14	-1.49
	WT-exclusive DEG		
MARCKSL1 **(MARCKS-related protein)**	Controls cell movement by regulating actin cytoskeleton homeostasis and filopodium and lamellipodium formation. When unphosphorylated, induces cell migration (By similarity). When phosphorylated by MAPK8, induces actin bundles formation and stabilization, thereby reducing actin plasticity, hence restricting cell movement, including neuronal migration (By	2.08	0.48

	similarity). May be involved in coupling the protein kinase C and calmodulin signal transduction systems (By similarity).		
FAM117A (Protein FAM117A)	N/A	2.34	-0.42
X.17116	N/A	4.90	1.57



Supplementary Figure 4.3. Western blot analysis of the JNK2 protein in PMM2-CDG and WT skin fibroblasts. Fibroblasts were stimulated with 10 ng/ml of TNF-α for increasing periods of time. Immunoblotting was performed using anti-JNK2 (Cell Signaling) at 1:1000. Mouse monoclonal anti-α-tubulin staining was performed as loading control.

CHAPTER 5.

SUPPLEMENTARY MATERIAL

Supplementary Table 5.1. Semi-structured interview guide.

Section	Step	Description
Introduction	1	Interviewer introduction
	2	Project summary.
	3	Research objectives and aim of interview. Explanation that interviewee may interrupt whenever there is need to clarification.
	4	Call settings (confidential, not recorded but notes will be taken, open-end questions, prediction of 60 minutes)
	5	Importance of the collection of this type of data.
CDG symptoms impact	6	What is your (parents of children and adults with CDG) experience with [SIGN/SYMPTOM]?
	7	What are the perceived consequences of the [SIGN/MANIFESTATION] on your child or on the family dynamics? (OR) What is the real meaning or impact of [SIGN/MANIFESTATION] on your child or your family daily life? (OR) In what way does having [SIGN/MANIFESTATION] impacts on your quality of life? List of SIGNS/MANIFESTATIONS on the data collection sheet (Supplementary File 2)
Closing interview	8	Acknowledgment
	9	Availability of results
	10	Farewell

Supplementary Table 5.2. Demographics of the 7 PMM2-CDG patients included in proxy qualitative interviews.

Patient	Country	Age (years old)
1	Portugal	40
2	Portugal	5
3	Switzerland	7
4	Portugal	6
5	Netherlands	25
6	United Kingdom	10
7	Spain	10

Supplementary Table 5.3. Groups of keywords used to build the search query. Keywords within the group were connected using the Boolean operator "OR" while the different groups were connected using the operator "AND". For some signs and symptoms, the keywords AND ("metabolic" OR "rare disease") were added to the combination to provide more specific results.

Signs and symptoms keywords	(AND) Patient reported outcomes keywords	(AND) Quality of Life keywords
("Behavioral problems" OR "Aggressive behaviour")	("patient reported outcomes"	("quality of life"
("Developmental delay" OR "Failure to thrive" OR "Gross motor delay" OR "Fine motor	OR "PRO"	OR "health related quality of life"
delay" OR "psychological functioning" "Social development" OR "Social functioning"	OR "patient reported outcome measure"	OR"HRQOL"
OR "social isolation" OR "emotional development" OR "emotional functioning" OR	OR "PROM"	OR "disease-specific quality of life")
"Intellectual disability" OR "Learning disability" OR "Mental retardation")*	OR "observer reported outcomes"	
(Dysphagia OR "Tube feeding" OR "Enteral nutrition" OR Malabsorption OR	OR "observer reported outcome measure"	
Enteropathy OR Diarrhea OR vomiting)*	OR "patient centered outcomes"	
(Dysarthria OR "Speech delay" OR "Language delay")	OR "patient centred outcomes"	
Hypotonia	OR "proxy reported outcomes"	
Infections*	OR "survey"	
(Kyphosis OR Scoliosis)*	OR "questionnaire")	
(Strabismus OR "Retinitis Pigmentosa" OR Esotropia OR nystagmus OR myopia)		
Ataxia		
Osteopenia*		
"Peripheral neuropathy"*		
(Seizures OR epilepsy OR convulsions)*		
Stroke-like episodes		
Coagulopathy*		
("Sleep disturbances" OR insomnia)*		
"Food allergy"*		
("hypergonadotropic hypogonadism" OR "delayed puberty" OR "incomplete puberty"		
OR "absent puberty" OR amenorrhea)		
(hepatomegaly OR "fatty liver" OR steatosis OR Cirrhosis OR "Liver failure")*		

Supplementary File 5.1. Quality analysis of the included PROMs or ObsROMs specific for the identified impactful PMM2-CDG signs/symptoms (.xlsx). The psychometric properties (namely Content, Criterion and Construct Validity, Internal Consistency, Agreement, Reliability, Responsiveness, Floor and Ceiling effect and Interpretability) of the identified PROMs or ObsROMs described on the original development and/or validation articles of the instruments were evaluated based on the adapted Quality Criteria for Measurement Properties of Health Status Questionnaire developed by Terwee et al., (2007) [2]. Based on these criteria, each property was evaluated with (+) - positive rate; (?) - indeterminate or doubtful rate; (-) - negative rate; or (0) - no information available. Available at https://bit.ly/PROQualityAnalysis.

Supplementary File 5.2. Summary results of the interviews encompassing the experiences with impactful PMM2-CDG clinical manifestations (.xlsx). Summary of the insights about the real-word impact of the signs and symptoms identified in the survey as being "the most impactful" from families' perspectives, following the application of the guide described in Supplementary Table 5.1. Available at https://bit.ly/InterviewsQoL.

Supplementary Table 5.4. List of references for the included symptom/disease-specific quality of life tools.

Symptom/disease-specific quality of life tool	References
10-item Neuro-Ophthalmic Supplement (NOS) to the NEI-VFQ-25	● Ihl T, Kadas EM, Oberwahrenbrock T, Endres M, Klockgether T, Schroeter J, et al. Investigation of Visual System Involvement in Spinocerebellar Ataxia Type 14. Cerebellum. 2020;19(4):469-482. ● Kedar S, Ghate D, Murray EL, Corbett JJ, Subramony SH. Vision related quality of life in spinocerebellar ataxia. J Neurol Sci. 2015;358(1-2):404-8.
8-question QoL live interview developed by Kothari M et al., (2009)	● Kothari M, Balankhe S, Gawade R, Toshnival S. Comparison of psychosocial and emotional consequences of childhood strabismus on the families from rural and urban India. Indian J Ophthalmol. 2009;57(4):285-8.
Adult Strabismus Quality of Life Questionnaire - 11 item (AS-11)	● Gothwal VK, Bharani S, Kekunnaya R, Chhablani P, Sachdeva V, Pehere NK, Narasaiah A, et al. Measuring Health-Related Quality of Life in Strabismus: A Modification of the Adult Strabismus-20 (AS-20) Questionnaire Using Rasch Analysis. PLoS One. 2015;10(5):e0127064. doi: 10.1371/journal.pone.0127064.
Adult Strabismus Quality of Life Questionnaire (AS-20)	● Hatt SR, Leske DA, Iezzi R Jr, Holmes JM. Binocular Interference vs Diplopia in Patients With Epiretinal Membrane. JAMA Ophthalmol. 2020;138(11):1121-1127. ● Wang JY, Leske DA, Hatt SR, Holmes JM. Diplopia after strabismus surgery for adults with nondiplopic childhood-onset strabismus. J AAPOS. 2019;23(6):313.e1-313.e5. ● Sim PY, Cleland C, Dominic J, Jain S. Investigation of factors associated with the success of adult strabismus surgery from the patient's perspective. J AAPOS. 2018;22(4):266-271.e3. ● Khanna CL, Leske DA, Holmes JM. Factors Associated With Health-Related Quality of Life in Medically and Surgically Treated Patients With Glaucoma. JAMA Ophthalmol. 2018;136(4):348-355. ● Sah SP, Sharma IP, Chaudhry M, Saikia M. Health-Related Quality of Life (HRQoL) in Young Adults with Strabismus in India. J Clin Diagn Res. 2017;11(2):NC01-NC04. ● Leske DA, Hatt SR, Liebermann L, Holmes JM. Lookup Tables Versus Stacked Rasch Analysis in Comparing Pre- and Post intervention Adult Strabismus-20 Data. Transl Vis Sci Technol. 2016;5(1):11. ● Tandon AK, Velez FG, Isenberg SJ, Demer JL, Pineles SL. Binocular inhibition in strabismic patients is associated with diminished quality of life. J AAPOS. 2014;18(5):423-6. ● Alam D, Khan AA, Bani SA, Sharma R, Amitava AK. Gain beyond cosmesis: demonstration of psychosocial and functional gains following successful strabismus surgery using the adult strabismus questionnaire adult strabismus 20. Indian J Ophthalmol. 2014;62(7):799-803.

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Osteoporosis Quality of Life Questionnaire (OQLQ)	• Measuring quality of life in women with osteoporosis. Osteoporosis Quality of Life Study Group. Osteoporos Int. 1997;7(5):478-87.
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Quality Of Life In Epilepsy (QOLIE-10)	● Sarangi SC, Kaur N, Tripathi M. Assessment of psychiatric and behavioral adverse effects of antiepileptic drugs monotherapy: Could they have a neuroendocrine correlation in persons with epilepsy?. Epilepsy Behav. 2019;100(Pt A):106439. ● Beran R, Berkovic S, Black A, Danta G, Dunne J, Frasca J, et al. AUStralian study of titration to effect profile of safety (AUSTEPS): high-dose gabapentin (neurontin) in partial seizures. Epilepsia. 2001;42(10):1335-9.
QOLIE-10-P	● Steinhoff BJ, Wendling AS. Short-term impact of the switch from immediate-release to extended-release oxcarbazepine in epilepsy patients on high dosages. Epilepsy Res. 2009;87(2-3):256-9.
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Quality of Life in Essential Tremor Questionnaire (QUEST)	● Degeneffe A, Kuijf ML, Ackermans L, Temel Y, Kubben PL. Comparing deep brain stimulation in the ventral intermediate nucleus versus the posterior subthalamic area in essential tremor patients. Surg Neurol Int. 2018;9:244. ● Mohammed N, Patra D, Nanda A. A meta-analysis of outcomes and complications of magnetic resonance-guided focused ultrasound in the treatment of essential tremor. Neurosurg Focus. 2018;44(2):E4.

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	● Carreon LY, Sanders JO, Diab M, Sucato DJ, Sturm PF, Glassman SD. The minimum clinically important difference in Scoliosis Research Society-22 Appearance, Activity, And Pain domains after surgical correction of adolescent idiopathic scoliosis. Spine (Phila Pa 1976). 2010;35(23):2079-83.
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CHAPTER 6.

SUPPLEMENTARY MATERIAL

Supplementary Table 6.1. Patients' and tumours' characteristics of the TNBC cohort of this study.

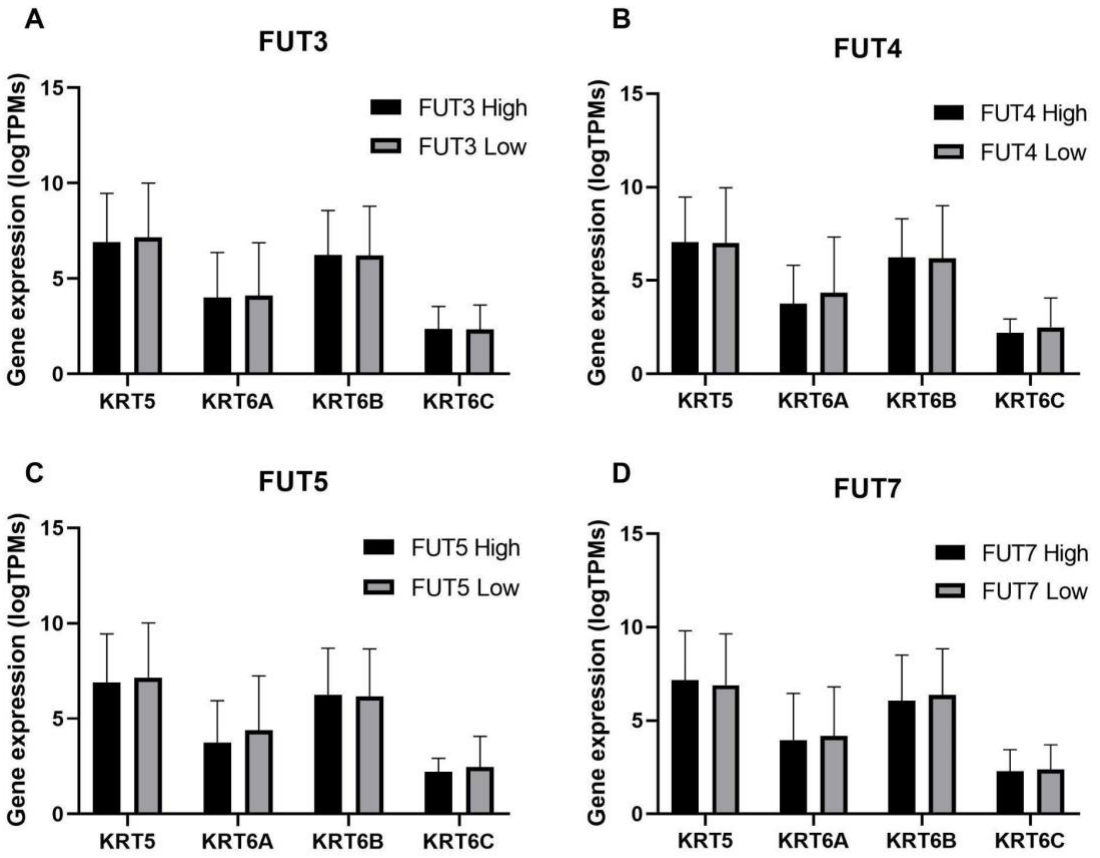
TNBC (n=50)	
Age at diagnosis (years)	
Median	64
Min	33
Max	89
Grade (% of cases)	
I	16%
II	26%
III	56%
IV	2%
Tumour size (mm)	
Median	15
Min	2
Max	70
# of invaded lymph nodes	
Median	12
Min	1
Max	25

Supplementary File 6.1. Complete clinical data of the TNBC patient cohort (.xlsx). Data includes: expression of $sLe^{X/A}$, E-SL; CK5/6; P-cadherin, EGFR and AR(%), age, tumour size, tumour grade, number of invaded lymph nodes, status of the patient, diagnosis date, disease date and ;last follow up date. Available at https://bit.ly/TNBCClinicalData.

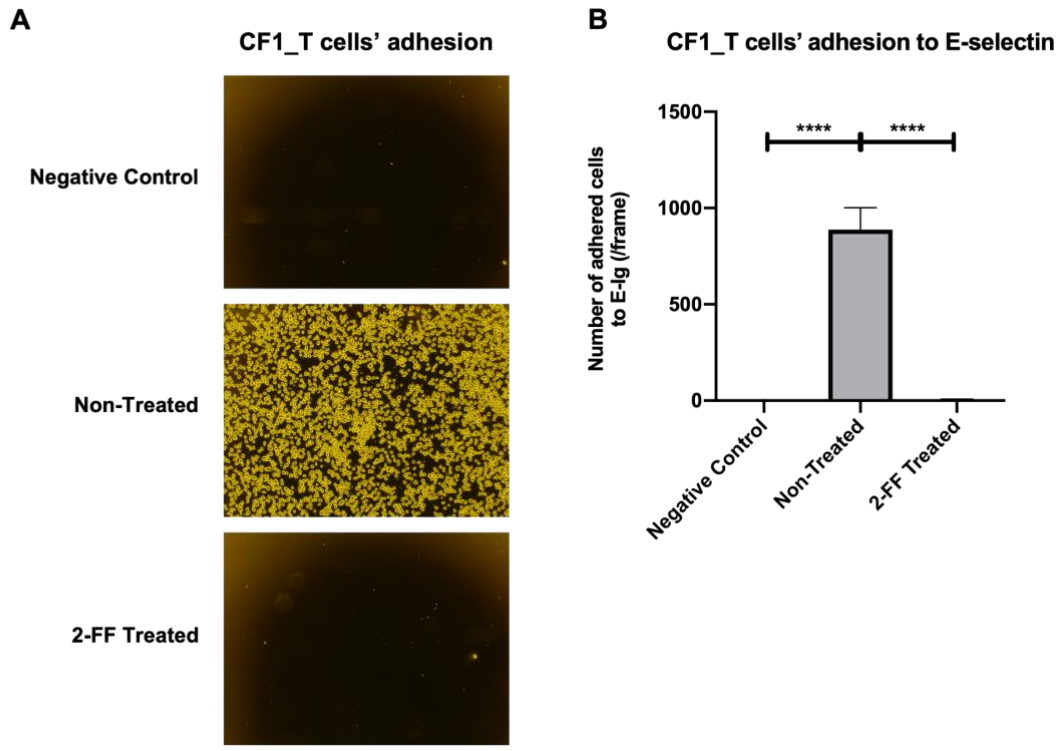
Supplementary File 6.2. R script with the survival analysis of TNBC patients (.txt). Available at https://bit.ly/RScriptSurvival.

Supplementary File 6.3. R script with the correlation analysis of $sLe^{X/A}$ with the other biomarkers and clinical features (.txt). Available at https://bit.ly/TNBCCorrelations.

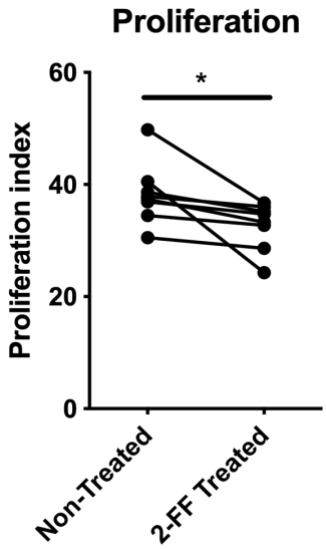
Supplementary File 6.4. R script to obtain Cytokeratin epitopes genes expression according to the high and low expression of fucosyltransferases genes (.txt). Available at https://bit.ly/CK_FUT.



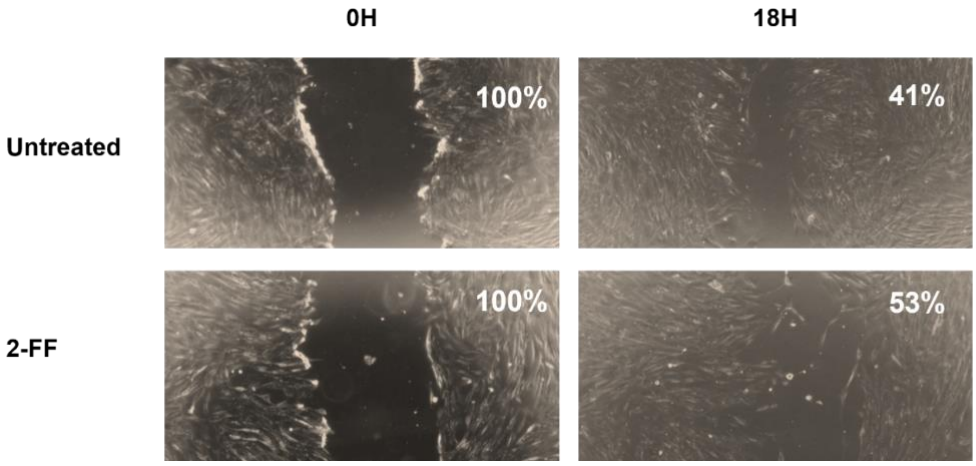
Supplementary Figure 6.1. CK5/6 genes expression according to the high or low expression of FUTs. TNBC tissue genetic data from the TCGA database was used to evaluate gene expression. The samples were subdivided based on the median expression of various 1,3-fucosyltransferases (*FUT3, FUT4, FUT5* and *FUT7*) and correlated with genes involved in cytokeratin production (*KRT5, -6A, -6B, -6C*).



Supplementary Figure 6.2. CF1_T cells treated with 2-FF lose the ability to bind E-selectin under flow conditions. (A) CF1_T were treated or not with 1 mM 2-FF for 5 days and resuspended in Hank's balanced salt solution (HBSS) containing 2 mM CaCl2 (HBSS-Ca). They were overlaid in glass slides spotted with E-Ig chimaera, previously blocked with 1% BSA. Their ability to bind to E-Ig chimaera was analysed under flow conditions, specifically using an orbital rotation at 80 r.p.m. for 30 min at 4 °C. The slides were immersed in HBSS-Ca to remove the non-adherent cells, and adherent cells were fixed with 3% glutaraldehyde. Assays performed in EDTA buffer were used as negative control. Adherent cells were examined under light microscopy at 100× magnification. The photomicrographic sections are a representative sample of a n = 4. (B) The number of adherent cells in each photomicrograph was counted using ImageJ software. [p<0.0001 (****)]

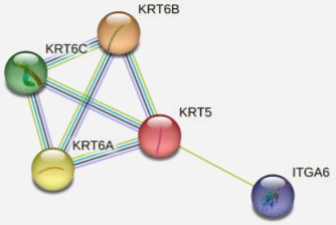
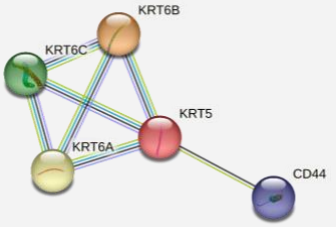
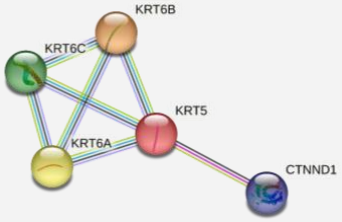
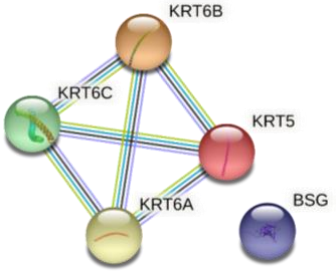
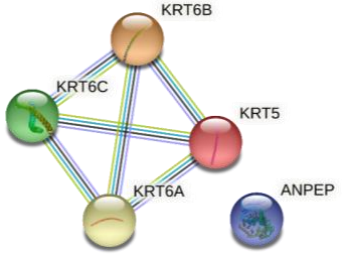


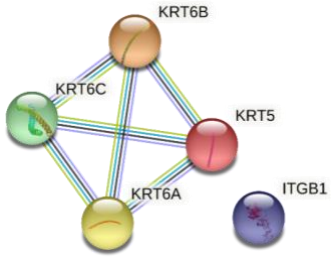
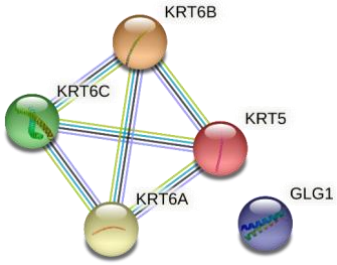
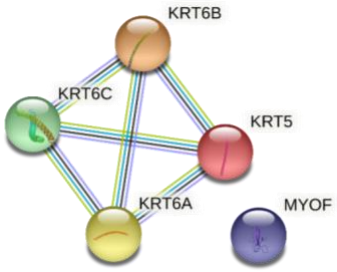
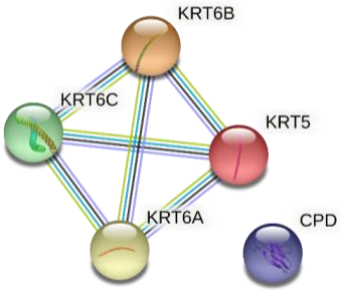
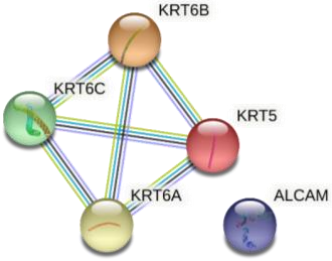
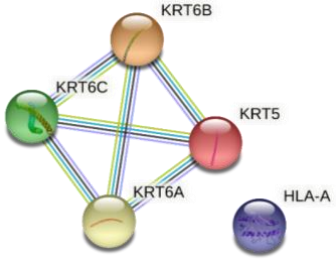
Supplementary Figure 6.3. Proliferation index of CF1_T cells treated with 2-fluorofucose (2-FF). CF1_T cells were treated, or not, with 2-FF, for a total of 14 days. At day 5 of treatment, the cells were stained with CFSE dye and then analysed after another 9 days of treatment (total of 14 days). CFSE dilution was analysed by flow cytometry, and the proliferation index was calculated as the fold expansion of the overall culture using the MODFIT software [n = 8; P < 0.05 (*)].

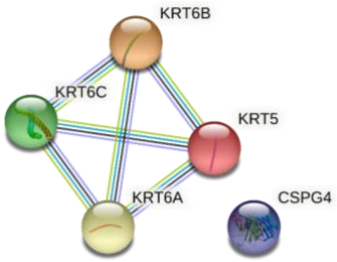
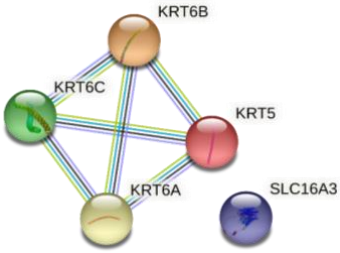
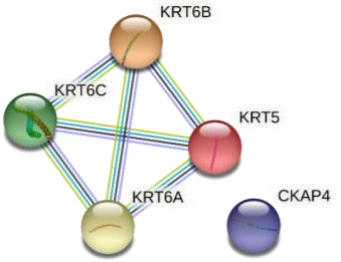
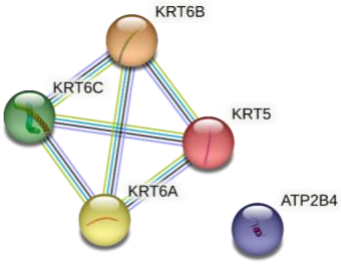
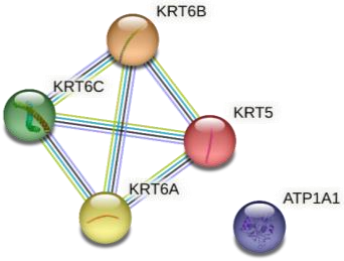
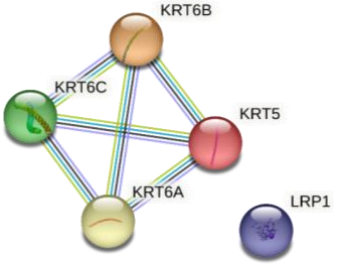


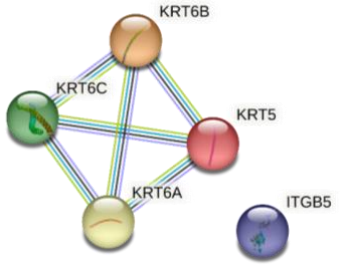
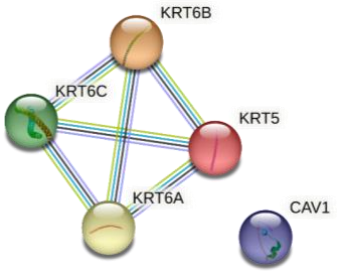
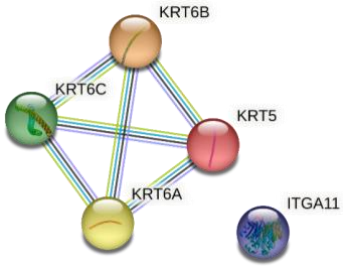
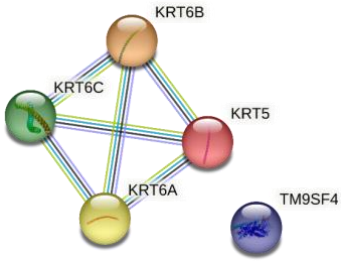
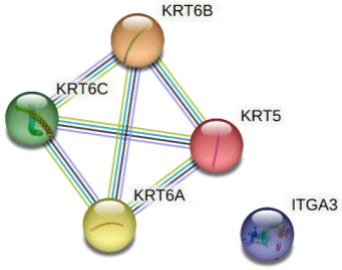
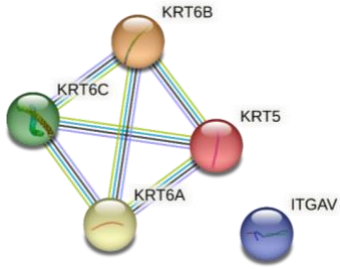
Supplementary Figure 6.4. Migratory ability of CF1_T cell line after 2-fluorofucose (2-FF) treatment analysed by scratch wound healing assay. CF1_T cells were seeded into 12-well microplates and grown to confluence in the presence or absence of 1mM of 2-FF inhibitor. After wounding the monolayer with a pipette tip. The suspended cells and debris were washed away, and fresh medium was added. At 0 and 18 h after wounding, scratched regions were photographed with an inverted microscope equipped with a digital camera. The percentage of wounded area in the photographs is indicated in white. It was measured using ImageJ software and the percentage of the closed area was calculated using the formula: ((wounded area (18h) / wounded area (0h)) *100) – 100)).

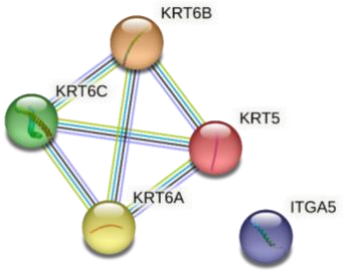
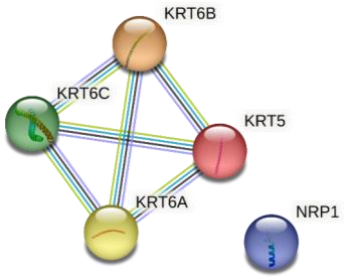
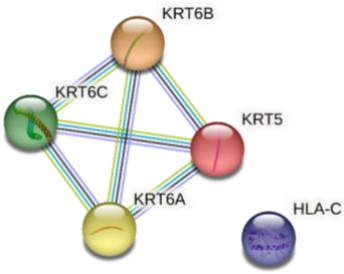
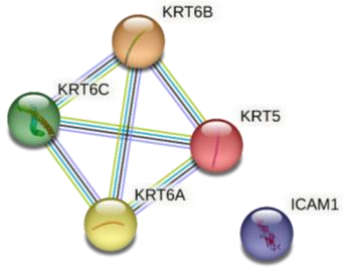
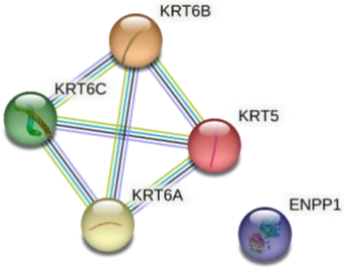
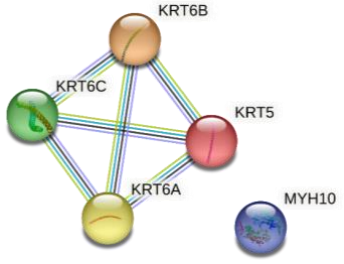
Supplementary Table 6.2. List of retrieved $sLe^{X/A}$ proteins immunoprecipitated from the CF1_T breast cancer cell line based on a label-free quantification of glycoproteins by mass spectrometry (as reported by Carrascal et al 2018 and their reported association with Cytokeratin 5/6. The association of each protein with cytokeratin 5/6 genes is annotated according to the STRING database for protein-protein interactions (Szklarczyk et al. Nucleic acids research 47.D1 (2018): D607-D613.2). When there is a positive association, STRING combined scores are annotated. *The combined score refers to the value of interaction between cytokeratin 5 and $sLe^{X/A}$ proteins. It accounts for co-expression, experimental/biochemical data, association in curated databases and co-mention in PubMed abstracts. This interaction is highlighted in the picture by a binding string. Proteins are presented by highest to lower combined scores.

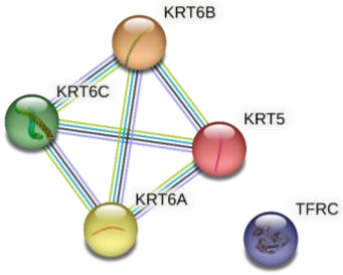
Entry	Entry name	Protein names	Positive association with cytokeratin	Combined Score*
P23229	ITA6_HUMAN	Integrin alpha-6 (CD49 antigen-like family member F) (VLA-6) (CD antigen CD49f) [Cleaved into: Integrin alpha-6 heavy chain; Integrin alpha-6 light chain; Processed integrin alpha-6 (Alpha6p)]		0.658
P16070	CD44_HUMAN	CD44 antigen (CDw44) (Epican) (Extracellular matrix receptor III) (ECMR-III) (GP90 lymphocyte homing/adhesion receptor) (HUTCH-I) (Heparan sulfate proteoglycan) (Hermes antigen) (Hyaluronate receptor) (Phagocytic glycoprotein 1) (PGP-1) (Phagocytic glycoprotein I) (PGP-I) (CD antigen CD44)		0.616
O60716	CTNND1_HUMAN	Catenin delta-1 (Cadherin-associated Src substrate) (CAS) (p120 catenin) (p120(ctn)) (p120(cas))		0.554
P35613	BASI_HUMAN	Basigin (5F7) (Collagenase stimulatory factor) (Extracellular matrix metalloproteinase inducer) (EMMPRIN) (Leukocyte activation antigen M6) (OK blood group antigen) (Tumor cell-derived collagenase stimulatory factor) (TCSF) (CD antigen CD147)		N/A
P15144	AMPN_HUMAN	Aminopeptidase N (AP-N) (hAPN) (EC 3.4.11.2) (Alanyl aminopeptidase) (Aminopeptidase M) (AP-M) (Microsomal aminopeptidase) (Myeloid plasma membrane glycoprotein CD13) (gp150) (CD antigen CD13)		N/A

P05556	ITB1_HUMAN	Integrin beta-1 (Fibronectin receptor subunit beta) (Glycoprotein IIa) (GPIIA) (VLA-4 subunit beta) (CD antigen CD29)		N/A
Q92896	GSLG1_HUMAN	Golgi apparatus protein 1 (CFR-1) (Cysteine-rich fibroblast growth factor receptor) (E-selectin ligand 1) (ESL-1) (Golgi sialoglycoprotein MG-160)		N/A
Q9NZM1	MYOF_HUMAN	Myoferlin (Fer-1-like protein 3)		N/A
O75976	CBPD_HUMAN	Carboxypeptidase D (EC 3.4.17.22) (Metallocarboxypeptidase D) (gp180)		N/A
Q13740	CD166_HUMAN	CD166 antigen (Activated leukocyte cell adhesion molecule) (CD antigen CD166)		N/A
P10314	1A32_HUMAN	HLA class I histocompatibility antigen, A-32 alpha chain (MHC class I antigen A*32)		N/A

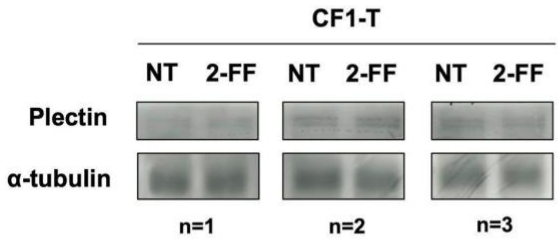
Q6UVK1	CSPG4_HUMAN	Chondroitin sulfate proteoglycan 4 (Chondroitin sulfate proteoglycan NG2) (Melanoma chondroitin sulfate proteoglycan) (Melanoma-associated chondroitin sulfate proteoglycan)		N/A
O15427	MOT4_HUMAN	Monocarboxylate transporter 4 (MCT 4) (Solute carrier family 16 member 3)		N/A
Q07065	CKAP4_HUMAN	Cytoskeleton-associated protein 4 (63-kDa cytoskeleton-linking membrane protein) (Climp-63) (p63)		N/A
P23634	AT2B4_HUMAN	Plasma membrane calcium-transporting ATPase 4 (PMCA4) (EC 3.6.3.8) (Matrix-remodeling-associated protein 1) (Plasma membrane calcium ATPase isoform 4) (Plasma membrane calcium pump isoform 4)		N/A
P05023	AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1 (Na(+)/K(+) ATPase alpha-1 subunit) (EC 3.6.3.9) (Sodium pump subunit alpha-1)		N/A
Q07954	LRP1_HUMAN	Prolow-density lipoprotein receptor-related protein 1 (LRP-1) (Alpha-2-macroglobulin receptor) (A2MR) (Apolipoprotein E receptor) (APOER) (CD antigen CD91)		N/A

P18084	ITB5_HUMAN	Integrin beta-5		N/A
Q03135	CAV1_HUMAN	Caveolin-1		N/A
Q9UKX5	ITA11_HUMAN	Integrin alpha-11		N/A
Q92544	TM9S4_HUMAN	Transmembrane 9 superfamily member 4 (Tumor cannibalism associated protein 1)		N/A
P26006	ITA3_HUMAN	Integrin alpha-3 (CD49 antigen-like family member C) (FRP-2) (Galactoprotein B3) (GAPB3) (VLA-3 subunit alpha) (CD antigen CD49c)		N/A
P06756	ITAV_HUMAN	Integrin alpha-V (Vitronectin receptor) (Vitronectin receptor subunit alpha) (CD antigen CD51)		N/A

P08648	ITA5_HUMAN	Integrin alpha-5 (CD49 antigen-like family member E) (Fibronectin receptor subunit alpha) (Integrin alpha-F) (VLA-5) (CD antigen CD49e)		N/A
O14786	NRP1_HUMAN	Neuropilin-1 (Vascular endothelial cell growth factor 165 receptor) (CD antigen CD304)		N/A
P10321	1C07_HUMAN	HLA class I histocompatibility antigen, Cw-7 alpha chain (MHC class I antigen Cw*7)		N/A
P05362	ICAM1_HUMAN	Intercellular adhesion molecule 1 (ICAM-1) (Major group rhinovirus receptor) (CD antigen CD54)		N/A
P22413	ENPP1_HUMAN	Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (E-NPP 1) (Membrane component chromosome 6 surface marker 1) (Phosphodiesterase I/nucleotide pyrophosphatase 1) (Plasma-cell membrane glycoprotein PC-1)		N/A
P35580	MYH10_HUMAN	Myosin-10 (Cellular myosin heavy chain, type B) (Myosin heavy chain 10) (Myosin heavy chain, non-muscle IIb) (Non-muscle myosin heavy chain B) (NMMHC-B) (Non-muscle myosin heavy chain IIb) (NMMHC II-b) (NMMHC-IIB)		N/A

P02786	TFR1_HUMAN	Transferrin receptor protein 1 (TR) (TfR) (TfR1) (Trfr) (T9) (p90) (CD antigen CD71)		N/A

A



B

Lane	Plectin intensity	α-tubulin intensity	Ratio plectin/α-tubulin
#2 NT	284.28	13450.86	0.02
#2 T	1845.84	11239.07	0.16
#3 NT	4436.86	17374.32	0.25
#3 T	4297.98	20084.22	0.21
#4 NT	3930.64	16042.87	0.24
#4 T	2280.38	13091.99	0.17

Supplementary Figure 6.5. CF1_T cell line expresses plectin. (A) CF1_T cell lines were treated with 2-FF inhibitor for 5 days and total lysate proteins were stained with α6 integrin antibody and analysed by western blot (n=3). α-tubulin expression was analysed as a loading control. (B) Densitometry readings of Western blot films regarding plectin and α-tubulin bands intensity. The ratios between plectin and α-tubulin intensities are also presented.

2023

CARLOTA MOUTINHO PASCOAL SMITH

TOWARDS THERAPEUTIC APPROACHES FOR HUMAN GLYCOSYLATION DISORDERS