NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF CHEMISTRY

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BSc in Biochemistry

ADDRESSING A GUT-SKIN AXIS THAT UNDERLIES A COMMON MECHANISM ASSOCIATED WITH THE IMMUNOPATHOGENESIS OF CROHN'S DISEASE AND HIDRADENITIS SUPPURATIVA

MASTER IN BIOCHEMISTRY FOR HEALTH

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“The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvellous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day.”

Albert Einstein

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ABSTRACT

Hidradenitis suppurativa (HS) is a chronic inflammatory skin disorder and one of the extraintestinal manifestations of Crohn's disease (CD). Although the association between both is not yet clarified, previous studies demonstrated a shared accumulation of anti-*Saccharomyces cerevisiae* (ASCA) antibodies, which raises relevant questions about a potential common immunopathogenesis of both inflammatory diseases, that we herein propose to tackle. Fibrocytes are a novel immune cell population and their role in chronic inflammation and autoimmunity has been described in several diseases. Glycans are complex and diverse structures decorating the cell surface and mediating a wide range of cellular and biological mechanisms. The glycome of each cell is unique, and the information it contains can be decoded by the immune system, which highlights glycans as major molecules in homeostatic and pathological conditions.

In this project, we revealed that HS and CD patients display a common abnormal accumulation of high-mannose *N*-glycans, together with a down-regulation of *MGAT1* and *MAN2A1* glycogenes. High titters of anti-Man_9 antibodies, similar to ASCA-IgGs, were detected in both HS and CD patients. We also demonstrated that anti-Man_9 antibodies from HS tend to cross-react with colonic proteins from CD, pinpointing shared glycoepitopes between the skin and the gut. This highlights the importance of anti-Man_9 IgGs in mediating common pathogenic mechanisms involved in a gut-skin axis. Our results also revealed that fibrocytes are equipped with mannose-sensing properties that, in the presence of its ligand, increase their antigen-presenting capacity. Using a glycoengineered mouse model susceptible to colitis ($VT1^{-/-}$ aging mice), we observed significantly lower levels of fibrocytes in the skin, suggesting their involvement in the recognition of mannose residues in the gut. Altogether, our results indicate that an abnormal glycosignature in HS and CD may induce the production of autoreactive anti-Man_9 IgGs important to establish a gut-skin axis, together with fibrocytes.

Keywords: Autoimmunity, *N*-glycosylation, ASCA antibodies, Fibrocytes, Gut-Skin axis.

RESUMO

A Hidradenite Supurativa (HS) é uma doença inflamatória crónica da pele e uma das manifestações extraintestinais da doença de Crohn (DC). Embora a associação entre ambas não esteja clarificada, alguns estudos demonstraram um aumento comum de anticorpos anti-*Saccharomyces cerevisiae* (ASCA), levantando a hipótese de uma imunopatogénese comum entre ambas as doenças, que nos propomos a abordar. Os fibrócitos são uma nova população imune e seu contributo em inflamação crónica e autoimunidade já foi descrito em diversas doenças. Os glicanos são estruturas complexas e diversas que decoram a superfície celular, mediando vários processos celulares e biológicos. O perfil de glicanos de cada célula é único e a informação nele contida pode ser descodificada pelo sistema imunitário, destacando os glicanos como moléculas importantes em condições homeostáticas e patológicas.

Neste projeto, demonstrámos que doentes com HS e DC apresentam um perfil anormal de *N*-glicanos enriquecidos em manose e a uma diminuição da expressão dos genes *MGAT1* e *MAN2A1*. Altos níveis de anticorpos anti-Man_9, semelhantes a ASCA-IgGs, foram detetados nestes doentes. Demonstrámos também que os anticorpos anti-Man_9 dos doentes com HS tendem a reagir de forma cruzada com proteínas do intestino dos doentes com DC, revelando a partilha de epítopos glicosilados entre a pele e o intestino e destacando a importância destes anticorpos em mediar mecanismos patogénicos comuns no eixo intestino-pele. Os nossos resultados indicam que os fibrócitos possuem recetores de manose e que, na presença do seu ligando, aumentam a sua capacidade de apresentação antigénica. Demonstrámos também que um modelo animal com suscetibilidade para colite ($VT1^{-/-}$), apresenta níveis significativamente reduzidos de fibrócitos na pele, sugerindo o papel destes no reconhecimento dos resíduos de manose no intestino. Concluindo, a alteração da glicosilação em HS e CD poderá induzir a libertação de anticorpos anti-Man_9 autorreativos, importantes para o eixo intestino-pele em conjunto com os fibrócitos.

Palavras-chave: Autoimunidade, *N*-glicosilação, anticorpos ASCA, Fibrócitos, eixo Intestino-Pele.

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ABBREVIATIONS LIST

ABC	Avidin-biotin-peroxidase complex
ACK	Ammonium-chloride-potassium
ALG	Asparagine-linked glycosylation
AMPs	Antimicrobial peptides
AOM	Azoxymethane
APCs	Antigen-presenting cells
ASCA	Anti-*Saccharomyces cerevisiae* antibodies
Asn	Asparagine
AUC	Area Under the Curve
BCR	B-cell receptor
BSA	Bovine serum albumin
CD	Crohn's disease
cDNA	Complementary DNA
CHU	Centro hospitalar universitário
CLRs	C-type lectin receptors
CRDs	Carbohydrate-recognition domains
CTLs	C-type lectins
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DC-SIGN	Dc-specific intercellular adhesion molecule-3-grabbing non-integrin
dNTPs	Deoxyribonucleotide Triphosphates
Dol-P	Dolichol phosphate
DSS	Dextran Sodium Sulphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
Fuc	Fucose
FVD	Fixable viability dye
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GBP	Glycan-binding proteins

GDP	Guanidine diphosphate
GlcNAc	*N*-acetylglucosamine
GM-CSF	Colony-stimulating factor
GNA	*Galanthus nivalis agglutinin*
GnT	*N*-acetylglucosaminyltransferase
GPI	Glycosylphosphatidylinositol
HC	Healthy controls
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HS	Hidradenitis suppurativa
IBD	Inflammatory bowel disease
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IHQ	Immunohistochemistry
IIM	Idiopathic Inflammatory myopathies
IL	Interleukin
ILCs	Innate lymphoid cells
IMID	Immune-mediated inflammatory diseases
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
KO	Knock-out
L-PHA	*Phaseolus vulgaris Leucoagglutinin*
Man	Mannose
MFI	Median intensity fluorescence
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MR	Mannose receptor
NETs	Neutrophil extracellular traps
NeuAc	*N*-acetylneuraminic acid
NHEJ	Non-homologous end joining
NK	Natural killer cells
OD	Optical density
ON	Overnight
OST	Oligosaccharyltransferase
OVA	Ovalbumin
P/S	Penicillin streptomycin
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSA	PBS 2%BSA
PBST	PBS 0.05%Tween

PBT	PBS 1%BSA 0.05%Tween
PFA	Paraformaldehyde
PMFS	Phenylmethylsulphonyl fluoride
poly-LacNAc	Poly-*N*-acetyllactosamine
Pro	Proline
PRR	Pattern recognition receptors
PV	Psoriasis Vulgaris
RA	Rheumatoid arthritis
RBCs	Red blood cells
RIPA	Radioimmunoprecipitation assay buffer
RT	Room temperature
RT-qPCR	Quantitative real-time polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SAP	Serum amyloid P
SDS-PAGE	Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis
Ser	Serine
Sia	N-acetylneuraminic acid
Sj	Sjogren's syndrome
SLE	Systemic lupus erythematosus
SNA	*Sambucus nigra lectin*
SpA	Spondyloarthropathy
SSc	Systemic sclerosis
TBS	Tris-Buffered Saline
TBST	TBS 0.05%Tween
TCL	Total cell lysate
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
Th	T helper cells
Thr	Threonine
TLR	Toll-like receptors
TMB	Tetramethylbenzidine
TNFa	Tumour necrosis factor-alpha
Treg	Regulatory T cells
UC	Ulcerative colitis
UDP	Uridine diphosphate
UP	UltraPure
WT	Wild type
α-Glc	Glycosidases
α-Man	Mannosidases
αSMA	Alpha-smooth muscle actin

1. INTRODUCTION

1.1. IMID Diseases

IMIDs, or Immune-Mediated Inflammatory Diseases, comprise a class of severely incapacitating chronic disorders, in which several organs can be affected by either acute or chronic inflammation.[1] The majority of IMIDs are autoimmune diseases, where the loss of immune tolerance results in the incapacity to distinguish self from nonself, with subsequent production of autoantibodies by highly reactive B cells.[2] Some examples of IMIDs are Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Inflammatory Bowel Disease (IBD), Idiopathic Inflammatory myopathies (IIM), Systemic sclerosis (SSc) Sjogren's syndrome (Sj), Hidradenitis Suppurativa (HS), among others. Although IMIDs share identical inflammatory pathways, usually resulting in inflammation due to an imbalance of the normal immunological response, they also present distinct characteristics, namely their clinical phenotype, age and sex dissemination, tissue distribution, therapeutic options and responses.[3]

The incidence of autoimmune diseases continues to grow worldwide at an alarming rate. A study, published this year in the scientific journal *The Lancet*, was conducted on the UK population to characterize the incidence and prevalence of the 19 most common IMIDs from 2000 to 2019. During this period, it was estimated that 978 872, in a total of 22 009 375 individuals, had been diagnosed with at least one IMID (around 4%). Interestingly, of those diagnosed, 63.9% and 36.1% were female and male individuals, respectively. Unfortunately, one in ten individuals is thought to be affected by IMIDs, and their incidence rate continues to sharply increase.[4]

The apparent sex gap between men and women is not a novelty, since autoimmune disorders are more likely to be developed by women (**Figure 1.1**).[5] Some authors suggest that this sex gap may arise from a complex interplay between hormones, due to the inconstant oestrogen levels that women experience in puberty, pregnancy and even menopause, X chromosomes and pregnancy, where the immune system must adapt to not reject the foetus as an unknown object.[6]

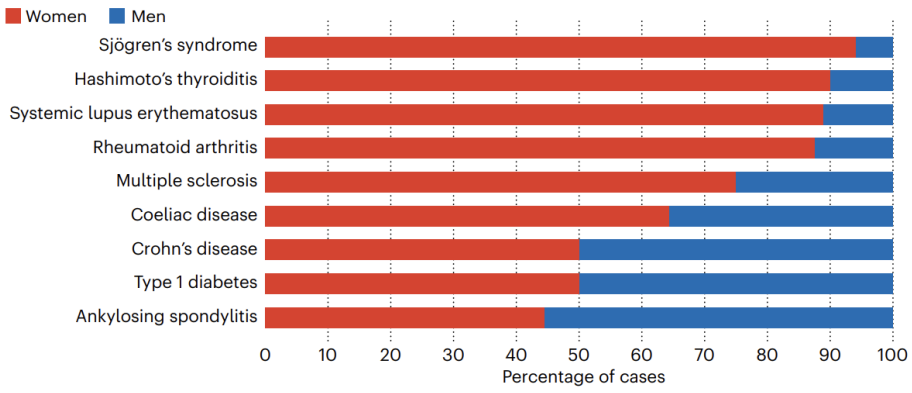

Figure 1.1 | | IMIDs are more prevalent in women than in men although, depending on the disease context, the sex differences may differ.[5]

Although IMIDs are still considered incurable, several advances have been made in this field in the past two decades. Indeed, with the development of monoclonal and molecular biotechnology approaches, we have transitioned from the application of a wide range of immune modulators (e.g., the use of glucocorticoids) to more precise and highly specific therapeutic agents, like pro-inflammatory cytokines inhibitors (e.g., tumour necrosis factor-alpha (TNF-α) in RA, and interleukin (IL)-17 in SLE).[3]

As asymptomatic preclinical stages usually precede IMIDs, there is an extreme urge to find early-onset disease biomarkers, that could assist early disease prediction. Therefore, further studies are required to better understand the immunopathogenesis of these disorders, explore new therapeutic targets and, ultimately, achieve disease remission and improve the quality of life of these patients, as they suffer from severe co-morbidities and mortality.

1.1.1. Hidradenitis Suppurativa (HS)

Hidradenitis suppurativa (HS) was first described by the French anatomist Alfred Velpeau in 1839, although it has been overlooked for a long time. HS is a chronic inflammatory skin disorder, commonly manifested by subcutaneous painful nodules, abscesses, pus secretion, fistula formation and disfiguring scars. This disorder, also known as *acne inversa*, is mostly manifested in apocrine glands-enriched areas like the axillae, groin, perigenital and perineal regions (**Figure 1.2.A**).[7] Apocrine glands are a key structural component of sweat glands, that usually develop closely to hair follicles (**Figure 1.2.B**), being composed of a subcutaneous secretory tubule and an excretory duct that drains sweat directly to the hair follicle.[8] Nowadays, HS is estimated to affect 0.05 - 4.10% of the worldwide population, with a higher incidence in young adults.[9] It is estimated that HS is twice as prevalent in women compared to men (73.8% women and 26.2% men in the USA), with a higher incidence in African Americans and biracial populations.[10]

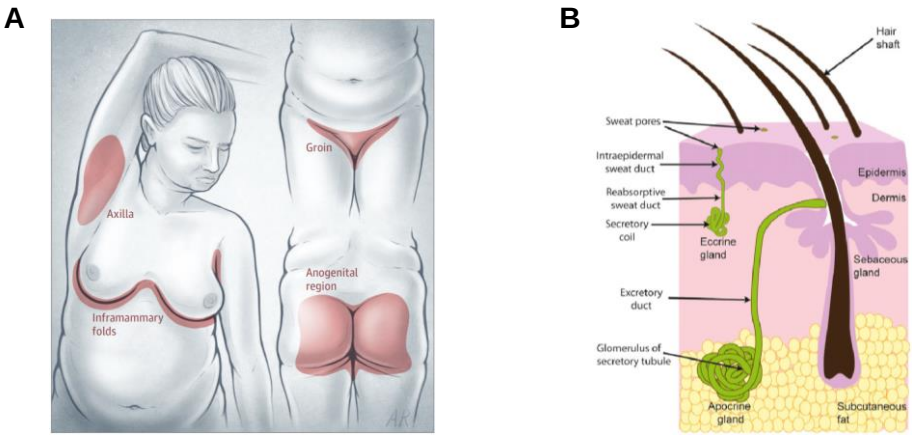

Figure 1.2 | Location and morphology of skin lesions in HS patients. (A) HS lesions are more predominantly manifested in apocrine glands-enriched areas: axillae, inframammary folds, groin and anogenital regions.[9] **(B)** The sebaceous glands are composed of eccrine and apocrine glands. The latest usually grows close to hair follicles, where the HS lesions are more predominantly.[8]

Despite the continuous efforts to better understand the aetiology of HS, several fundamental questions remain to be answered and explored, including the initial cause of this disorder. Nevertheless, it is known that the pathophysiology of HS is composed of three main stages, which can be triggered by a variety of endogenous and exogenous risk factors (**Figure 1.3**). The first event of the cascade includes the follicular occlusion by keratosis, usually characterized by an exacerbated production of keratin by epidermal keratinocytes. Subsequently, this follicular occlusion may result in the rupture of the hair follicle, leading to an abnormal immune response and the dissemination of pro-inflammatory cytokines, usually implicated in the activation of inflammatory responses. Ultimately, this may progress to inflammatory nodules and abscesses and, if a chronic inflammatory state is achieved, the formation of disfiguring scars and sinus tracts may be favoured.[11]

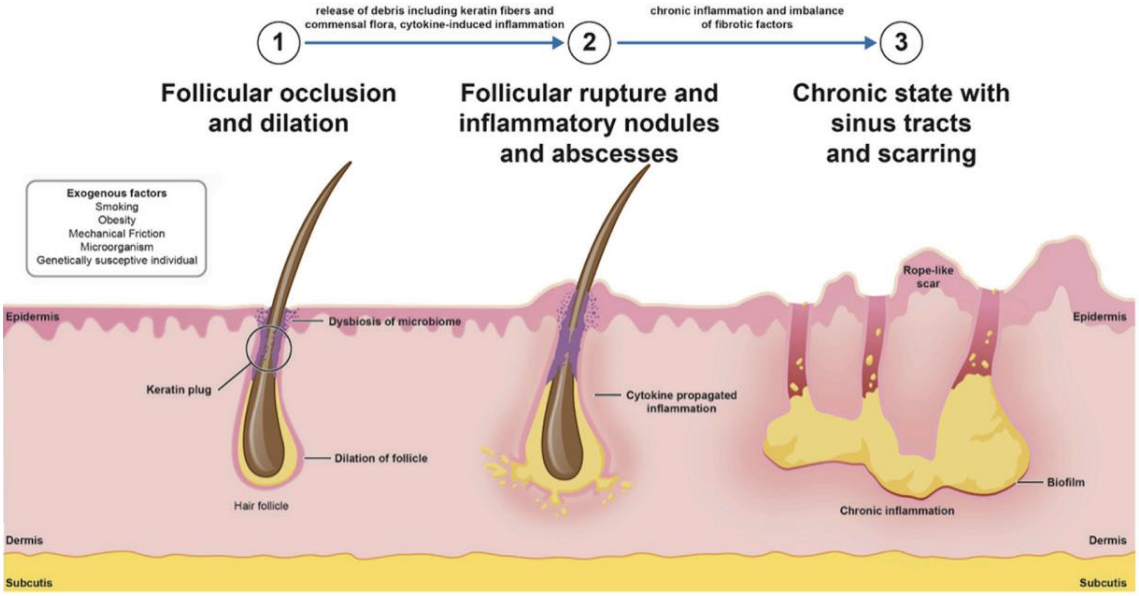

Figure 1.3 | The sequence of events regarding the HS pathophysiology is composed of three main stages. Firstly, the hair follicle is blocked, followed by its rupture and consequent inflammation propagation until a chronic inflammatory stage is reached.[11]

Histological lesions in HS skin are in accordance with the pathophysiology events above described (**Figure 1.3**). In fact, the HS lesions are markedly associated with epidermal thickness and hyperkeratosis, characterized by increased thickness of the stratum corneum, the skin's outer layer. Additionally, the hyperplasia of follicular epithelium and perifolliculitis (the accumulation of inflammatory cells within hair follicles) is also observed. Skin fibrosis, sinus tracts and stromal immune cell infiltration is also a histological finding of this disease (**Figure 1.4**).[12]

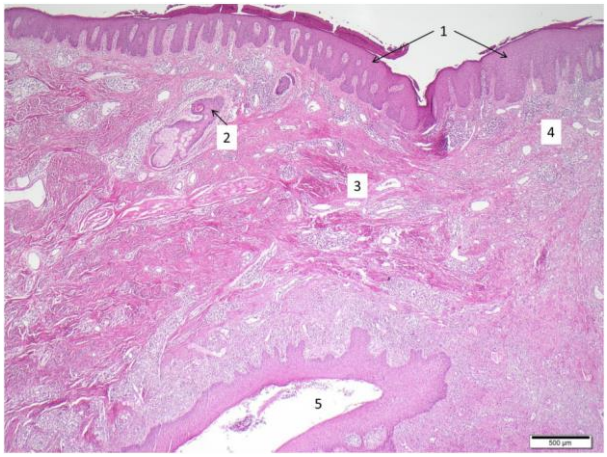

Figure 1.4 | Histological lesions in HS. (**1**) epidermal thickness and hyperkeratosis, (**2**) hyperplasia of follicular epithelium and perifolliculitis, (**3**) fibrosis, (**4**) immune cell infiltration and (**5**) sinus tracts.[12]

As already mentioned, several risk factors must be taken into account as potential triggers in the pathogenesis of HS, from genetic predisposition, imbalance of normal skin microbiota, immune dysregulation, exogenous factors (e.g., lifestyle) and Crohn's Disease (CD), as will be later detailed. The patient's lifestyle may include obesity, due to mechanical friction in skin folds, and smoking, since nicotine causes epidermal hyperplasia, resulting in hyperkeratosis and follicular blockage.[13] Additionally, and concerning genetic susceptibility, it is estimated that approximately 30% of HS cases have a family history, suggesting a dominant autosomal inheritance pattern.[14] Wang *et al*. [15] have identified γ-Secretase gene mutations in a wide range of Chinese families suffering from this condition. The γ-Secretase is a multi-subunit complex protease at the cell membrane, implicated in a variety of cellular processes and signalling cascades, including the Notch signalling pathway.[15] With this in mind, several authors have suggested a causal relationship between mutations in the γ-Secretase gene and lower activation of the Notch receptor, which may be important to the disease onset. Notch signalling pathway is important to regulate the fate of hair follicle stem cells and prevent their differentiation into keratinocytes, suggesting that its inactivation in the HS context may potentiate an abnormal proliferation of keratinocytes within hair follicles, leading to an increased risk for follicular occlusion.[16] Noteworthy, Pan *et al*. [17] have demonstrated that mice lacking γ-Secretase function in the skin, tend to develop epidermal and follicular cysts, similar to the HS histopathological lesions. Interestingly, some studies have already demonstrated the role of the Notch signalling pathway in the context of IBD.[18]

Unfortunately, patients suffer from severe co-morbidities that usually contribute to a decreased life quality and expectancy, like depression (associated with public stigma), metabolic syndrome, obesity, and spondyloarthropathy (SpA).[19] Afterwards, there is an extreme urge for an early and appropriate diagnosis of HS, to reduce or control the negative effects of this disease.

Concerning HS diagnosis, previous studies have demonstrated that it can take up to 7.2 years from the first manifestation of HS to its ultimate diagnosis, which may result from the medical establishment's lack of consciousness.[20] Thus, HS is often misdiagnosed or erroneously identified, and the consequent delay in initiating therapy may result in chronic pain, higher disease severity and morbidity. Despite the difficulty in diagnosing this condition, the diagnosis method may consider three key important branches: skin lesion morphology, location, and chronicity/recurrence.[21] Hurley's classification was the first system developed to categorize patients into different groups according to the severity of the disease. Currently, this system is still used, being very useful for a more accurate diagnosis and for the selection of a more suitable treatment. This classification comprises three clinical stages of HS: **Stage I** (single or multiple abscesses, with no sinus tracts and scars), **Stage II** (recurrent abscesses, accompanied by sinus tracts and scars, and several lesions in different locations), and **Stage III** (diffuse involvement, or a complex network of interconnecting sinus tracts and abscesses, spread across the lesion area).[7]

Regarding treatment, there are three main therapeutical options available for HS patients: topical/system antibiotics, biological agents, and surgery.[21] The use of antibiotics, whether topical or systemic, arises from the possibility of secondary bacterial infections in HS skin lesions. Oral tetracyclines are commonly prescribed to mild disease patients (stage I and II), as they inhibit neutrophil migration, chemotaxis, and matrix metalloproteinase.[22] Although their benefits, the use of these medications must be carefully considered, to avoid bacterial resistance that may impair the treatment. Biological agents take into account the pathogenesis of HS and are mostly focused on pro-inflammatory cytokines. In fact, previous studies have demonstrated that TNF-α, as in CD treatment, and interleukin IL-1β, IL-12/23, and IL-17 pro-inflammatory cytokines have an important role in the chronic inflammatory pathways of HS. Adalimumab, a recombinant human immunoglobulin G (IgG) anti-TNF-α, is the first biological agent approved by the US FDA against moderate to severe status of HS.[23] Surgical interventions, despite not healing the disease, are important to improve the lifestyle of these patients, by removing draining tunnels, inflamed tissue, and scars from their skin.[21] The appropriate therapeutic approach must be adequately selected, taking into account the severity of the disease, and, in certain circumstances, it may be reasonable to combine several therapeutic options.

Nevertheless, further studies are required to better understand the immunopathogenesis of HS, and ultimately, reach therapeutical success for HS patients, regardless of their disease severity.

1.1.2. Crohn's Disease (CD)

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders, affecting the gastrointestinal tract, and includes both Ulcerative Colitis (UC) and CD. While inflammation in UC patients is confined to the colonic mucosa, in CD may have several focal points, with the terminal ileum and colon being the most impacted areas. In CD, the inflammation may lead to asymmetrical and transmural lesions that can affect any portion of the gastrointestinal tract, culminating in fistulas or abscesses formation.[24,25]

Unfortunately, IBD is a lifelong disabling condition, mainly diagnosed in young adults and its prevalence is estimated to remarkably increase in the upcoming decades.[26] In fact, the prevalence of IBD continues to grow worldwide with a higher incidence in industrialized countries (**Figure 1.5**), more specifically in Europe (322 per 100 000 individuals), Canada (319 in 100 000 individuals) and the USA (214 in 100 000 individuals), reflecting an estimated global incidence of 0.3%.[27]

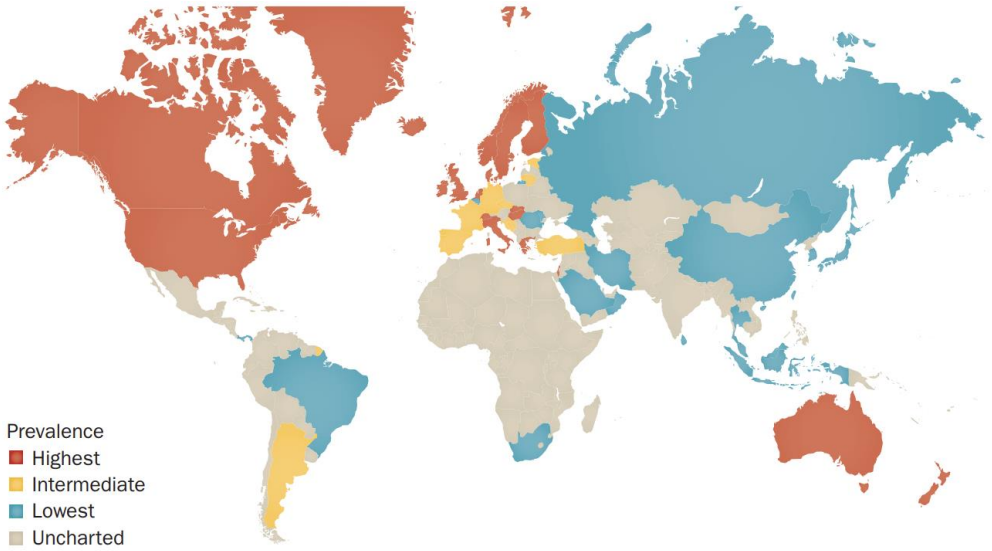

Figure 1.5 | Global prevalence of IBD in 2015. Developed countries show higher disease prevalence (USA, Canada and North Europe).[26]

Although the aetiology of CD is not fully understood, it can be classified as a multifactorial disorder since multiple risk factors are believed to have an impact on the development of this illness. Some of them may include genetic predisposition, environmental factors, lifestyle, and intestinal microbiome composition, among others. These risk factors play an essential role in activating an exacerbated immune response, leading to the loss of gut homeostasis and the impairment of epithelial barrier function, which enables intestinal lumen contents to reach the lamina propria.[28]

Unfortunately, these patients suffer from severe co-morbidities like abdominal pain, rectal bleeding, chronic and bloody diarrhoea, weight loss, anorexia and fatigue that severely impact their life quality.[25] Sometimes, CD can evolve to a more complicated phenotype, associated with structuring and penetrating lesions usually requiring surgical intervention, due to young disease onset, ileal location, rectal disease, and perianal fistulas.[29]

Patients with CD experience a wide range of symptomatological features, making the diagnosis of this condition very demanding. Nevertheless, the diagnosing criteria may take into account the symptoms and physical abnormalities, requiring both radiographic and endoscopic techniques and the corresponding pathological findings (focal, asymmetric, and transmural lesions).[30] After receiving the proper diagnosis, patients are categorized following the Montreal classification system, accordingly to three important parameters: age at disease onset, disease location and severity.[31]

Therapeutical strategies against CD are selected taking into consideration disease severity and it has two important stages: induction and maintenance periods. Typical therapeutical agents include corticosteroids (to control immune system response), immunosuppressants (usually prescribed in the maintenance phase, these medications, like thiopurines, are described to maintain disease remission and reduce the need for surgical interventions), biological agents (anti-TNF-α agents, like adalimumab, are selected in patients that failed to respond to either steroids or immunosuppressants drugs) and surgery, in the most clinically severe cases.[25]

Although several advances have been made to improve disease diagnosis and treatment, the prediction capacity of CD is still very challenging and limited. In fact, risk factors are poorly accurate in predicting the outcome of this disorder, leading to an increasing demand for the identification of novel serological biomarkers. Afterwards, a study conducted by Torres and Petralia *et al*. [32] demonstrated that high titters of anti-*Saccharomyces cerevisiae* antibodies (ASCA IgG or IgA) can be detected in individuals that will develop CD within 5 years, therefore being considered as an important biomarker. Moreover, the presence of these serological antimicrobial antibodies several years before the diagnosis can be linked to a more complicated disease phenotype (structuring and penetrating lesions) at the diagnosis onset.[33,34]

Similar to other IMIDs, these studies have shown that an asymptomatic phase usually precedes CD, a stage in which disease-specific biomarkers can be accurately identified, being useful in a clinical scenario for an early disease diagnosis and, ultimately, prevention.

1.1.3. Association between HS and CD

As previously stated, HS has been associated with a variety of clinical conditions and syndromes, including metabolic syndrome, depression, SpA, and CD, which raise questions about the similarities between HS and CD. In fact, previous studies have already demonstrated that CD is 8 times more prevalent in patients with HS (2.5%, in a total of 1076 HS patients) than in the general population, in which the estimated global incidence is 0.3%.[35] Accordingly, it has also been described that up to 26% of patients suffering from CD have HS.[36] Altogether, these data pinpoints that HS patients display an increased risk for developing CD, and vice-versa.

Despite the continuous efforts to better clarify the pathogenesis of both HS and CD, no clear mechanism linking these two has yet been identified, although some authors have suggested that this causal relationship may be explained by common clinical manifestations in HS and CD patients. In fact, these patients may suffer from perianal lesions, although in CD these lesions are associated with a more complicated disease phenotype, where the lesions are more ulcerative and restricted to the skin near the anus. Additionally, patients' lifestyles (e.g., smoking is a well-established risk factor for both CD and HS), genetic susceptibility and abnormal microbiome composition, that may compromise the epithelial homeostasis of both skin and gut, by the activation of pathogen recognition receptors, like Toll-like receptors (TLR), may also associate both IMIDs. Finally, common deregulatory immune pathways implicated in the pathogenesis of these disorders may also be considered (e.g., common pro-inflammatory cytokines are implicated in the pathogenesis of HS and CD, such as TNF-α and IL-17, as will be later detailed).[7,37]

Moreover, Assan *et al.* [38] conducted a study from 2012 to 2017 in France, to investigate the prevalence of ASCA antibodies in the serum of HS, including Hurley stage I (25,7%), II (37.2%), and III (37.2%) patients, and psoriasis vulgaris (PV). Significantly high titters of serological ASCA IgG and/or IgA were found in HS patients, in comparison with either PV or healthy subjects, suggesting ASCAs to be targeted as potential biomarkers of HS. It is important to highlight that these high levels of ASCAs were predominantly identified in those with a more severe status of HS (Hurley stage III). Interestingly, and as previously mentioned, ASCA antibodies were also identified as a biomarker for CD, and its prevalence has also been associated with a more severe phenotype of CD and early disease onset.[32,39]

Altogether, these findings may suggest ASCA as a potential common link between HS and CD, raising the hypothesis of acting as a potential vehicle in the establishment of a novel gut-skin axis in CD and HS. Nevertheless, this topic remains poorly elucidated being the long-term goal of this Master thesis.

1.2. Immune System

The immune system is a complex and dynamic network of cells, molecules and pathways, capable of protecting our bodies against external antigens/pathogens while maintaining the ability to discriminate the self from the nonself (immune tolerance). Depending on antigen specificity and activation time, the immune system is composed of two interconnected branches: innate and adaptive immunity.[40] Immunity also includes both humoral and cell-mediated responses that are crucial to establish an immune response. Humoral immunity is mostly composed of soluble molecules in the blood serum, like antibodies, cytokines (pro or anti-inflammatory cytokines, important to cell signalling), chemokines (recruitment of immune cells to the site of infection) and complement, whereas cell-mediated immunity relates to the capacity of the immune system to produce highly effective immune cells against infection (e.g., lymphocytes).[41]

Innate immunity is a more primitive system that serves as the initial line of defence towards an invading pathogen, establishing a pro-inflammatory response to avoid the infection. This system is quickly activated after encountering the antigen and, since it is antigen-independent, there is no immunological memory for subsequent invasions. Innate immunity implies physical (skin and mucous membranes) and physiology (temperature, acid stomach pH) barriers, alongside immune cells (e.g., macrophages, neutrophils, natural killer and dendritic cells) and "pattern recognition receptors" (PRRs, such as the Toll-like receptors), essential for the recognition of "pathogen-associated molecular patterns" (PAMPs) or "damage-associated molecular patterns" (DAMPs).[42]

Macrophages are tissue-resident immune cells spread across the body with phagocytic activity, important for the engulfment and elimination of bacteria. These cells are capable of secreting cytokines, important for the immune response and the recruitment of other immune cells towards the infection site. According to the secreted cytokines, macrophages can be classified into M1 or M2 macrophages. M1 macrophages are usually activated by the colony-stimulating factor (GM-CSF) and interferon-gamma cytokine (IFN-γ), resulting in the production of pro-inflammatory cytokines (e.g., IL-1β, IL-6, IL-12, IL-23 and TNF-α). M2 macrophages are polarized by IL-4 and IL-13 acquiring anti-inflammatory properties characterized by anti-inflammatory cytokines (IL-10 and transforming growth factor beta (TGF-β)) and the downregulation of major histocompatibility complex (MHC) class II, important for initiating an immune response.[43]

Neutrophils are also phagocytic cells commonly known for the production of neutrophil extracellular traps (NETs), in which the pathogen loses its capacity to spread, therefore being important to prevent bacterial and fungal dissemination in the body.[44] Natural killer (NK) cells are a subtype of innate lymphoid cells (ILCs), important in immune responses against viruses and tumour cells. NK secret IFN-γ and TNF-α pro-inflammatory cytokines and have similar features to those of $CD8^+$ cytotoxic T cells. Lastly, NK cells present lytic granules enriched in perforin and granzyme B, responsible for the NK cell-mediated killing.[45]

Dendritic cells (DC), or antigen-presenting cells (APCs), are a unique subtype of immune cells that mediates the link between the innate and adaptive immune system. These cells are capable of recognising and presenting external antigens (PAMPs or DAMPs) to naïve T lymphocytes, culminating in the activation of T cells. This process is carried out in secondary lymphoid organs (lymph nodes), being mediated by the MHC, present at the surface of DCs. Furthermore, DCs also secrete a wide range of cytokines which mediate the differentiation of T cells into distinct effector T cell phenotypes.[46,47]

Adaptive immunity is a mechanism that proceeds the innate immunity, capable of distinguishing the "self" from the "nonself" and generating antigen-specific responses mainly mediated by T and B lymphocytes, both involved in humoral and cell-mediated immunity. This system is recruited when innate immunity fails to remove the infection and, contrary to innate responses, it generates immunological memory to protect our body against future infections.[48]

T lymphocytes are developed in the thymus and express the T-cell receptor (TCR) on their surface. According to the TCR they present, these cells can be divided into two groups: TCR $\alpha\beta^+$ or TCR $\gamma\delta^+$ ($\gamma\delta$ T cells). The TCR $\alpha\beta^+$ interacts with peptide antigens presented by class I or II MHC, resulting in different subsets of T cells: $CD4^+$ T cells (MHC class II-restricted cells) and $CD8^+$ T cells (MHC class I-restricted cells, expressing cytotoxic granules that induce the death of infected target cells). Moreover, $CD4^+$ T cells may differentiate into regulatory T cells (Treg), with anti-inflammatory properties (TGF-β and IL-10 cytokines), or T helper cells (Th) which vary between Th1, Th2, and Th17, depending on the secreted cytokines.[49] B lymphocytes are derived from bone marrow stem cells and provide long-term protection against foreign antigens. After receiving the proper stimuli, B cells can evolve into antibody-secreting plasma cells, where the antibodies recognize and neutralize the external antigen, or in memory B cells. Contrary to T cells, B cells can directly recognize an external antigen, without the need for an APC, through highly specific Ig on their surface, commonly known as B-cell receptors (BCR).[50]

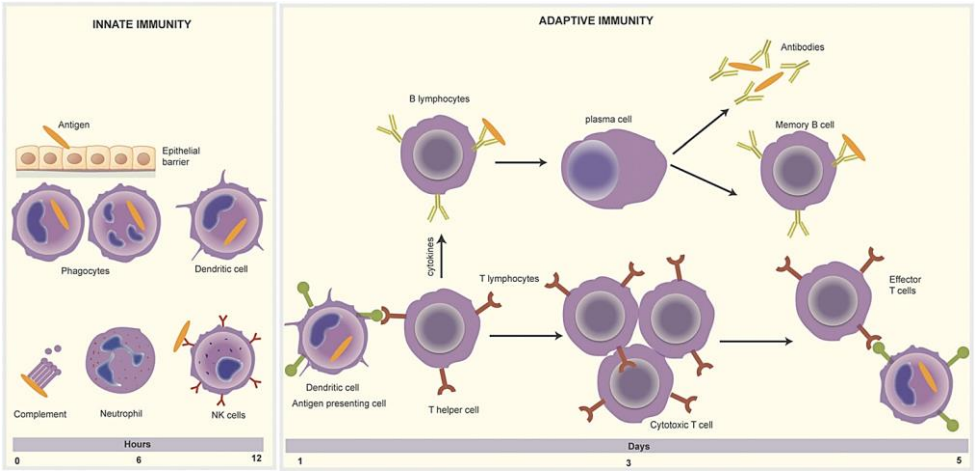

Figure 1.6 | The fundamental elements of both innate and adaptive immune systems in response to an external pathogen. The figure represents the most important immune cells within the innate and adaptive immune system, as well as the period in which these responses are activated.[51]

1.2.1. The loss of immune tolerance in IMIDs

The main purpose of the immune system is to protect the host from foreign pathogens, without harming self-tissues. This ability is commonly referred to as immune tolerance, which allows the discrimination between self from nonself and the subsequent immune response against unknown antigens.[40]

The immune tolerance is mainly ensured by both T and B lymphocytes, particularly by their TCR and BCR, which have the capacity to recognize an antigen and, if it is detected as a nonself object, initiate an adaptive immune response towards its elimination (e.g., the production of antibodies by plasma B cells, with high affinity to that antigen). With this in mind, T and B lymphocytes must harbour an extensive and diverse network of TCR and BCR specificities to respond to the huge diversity of microorganisms or abnormal host cells that the host may encounter during its life. In fact, during the development of T and B lymphocytes, DNA recombination events take place, by a process called V(D)J recombination, to generate random recognition receptors.[52,53] Briefly, V(D)J recombination refers to a random rearrangement of the variable (V), sometimes the diversity (D) and joining (J) segments of the genes encoding for TCR and BCR, resulting in a huge diversity of the antigen-binding regions of these receptors. The RAG 1 and 2 recombinase proteins are the main players in this process, as they specifically cleave DNA sequences surrounding each V, D and J segments. The final steps require the traditional non-homologous end joining (NHEJ) method, to join the DNA sequences.[54,55]

Although such diversity is required for a functional immune system, in some cases, receptors with host epitope specificities can be generated, increasing the risk of loss of immune tolerance. To prevent this, the immune system is equipped with central and peripheral mechanisms that ensure the inactivation or removal of T and B cells with high affinity to host epitopes. The central tolerance is responsible for the negative selection of auto-reactive T and B lymphocytes during their development stages in the thymus and bone marrow (primary lymphoid organs), respectively. Only the cells that show little or no affinity to self-antigens will successfully pass this first test, entering the secondary lymphoid organs (lymph nodes and the spleen), where the peripheral tolerance takes place. In fact, this second mechanism allows the inhibition of self-reactive T and B cells by three important processes: anergy (loss of function response), deletion (by apoptosis) and suppression by Tregs.[56,57]

Unfortunately, these mechanisms are not completely effective, and sometimes self-reactive T and B lymphocytes are not correctly identified and eliminated, which compromises immune tolerance. Therefore, the discrimination between self from the nonself is no longer possible and the immune system starts to attack the host, culminating in the high titters of autoantibodies and pro-inflammatory cytokines that establish an autoimmune response. Thus, the loss of self-tolerance is directly associated with autoimmune diseases, where the immune system transforms into self-damage.[58]

1.2.2. Immunopathogenesis of HS

In recent years, several efforts have been employed to better understand the immunopathogenesis of HS however, the current knowledge is still limited and requires further exploitation. Nevertheless, the mechanism underlying HS includes an abnormal and extensive activation of the immune system and the consequent progression to a chronic inflammatory disease phenotype.[59]

As already mentioned, the initial events regarding the pathogenesis of this disorder (**Figure 1.7**) include the follicular occlusion by hyperkeratosis and the subsequent dilatation of the hair follicle and bacterial propagation. Consequently, this bacterial content and DAMPs can be recognized by skin-resident macrophages through TLR, resulting in the secretion of pro-inflammatory cytokines like TNF-α and IL-1β. The latest is described to interact with fibroblasts, resulting in the production of chemokines (e.g., CXCL1 and CXCL6) that further recruit more immune cells to the inflammation site.[60] Similarly, TNF-α may also induce the expression of chemokines by keratinocytes that attract more neutrophils, T cells (e.g., Th1 and Th17), B cells and monocytes that can later differentiate into macrophages or DCs. The role of B cells in the immunopathogenesis of HS remains unexplored, despite their relevance in the production of autoantibodies (e.g., ASCA antibodies [38]), a well-established hallmark in IMIDs. Afterwards, high levels of chemokines and the activation of endothelial cells in blood vessels lead to an abnormal immune cell infiltration that ultimately contributes to a chronic inflammatory state and the morphological skin lesions of HS patients.[61]

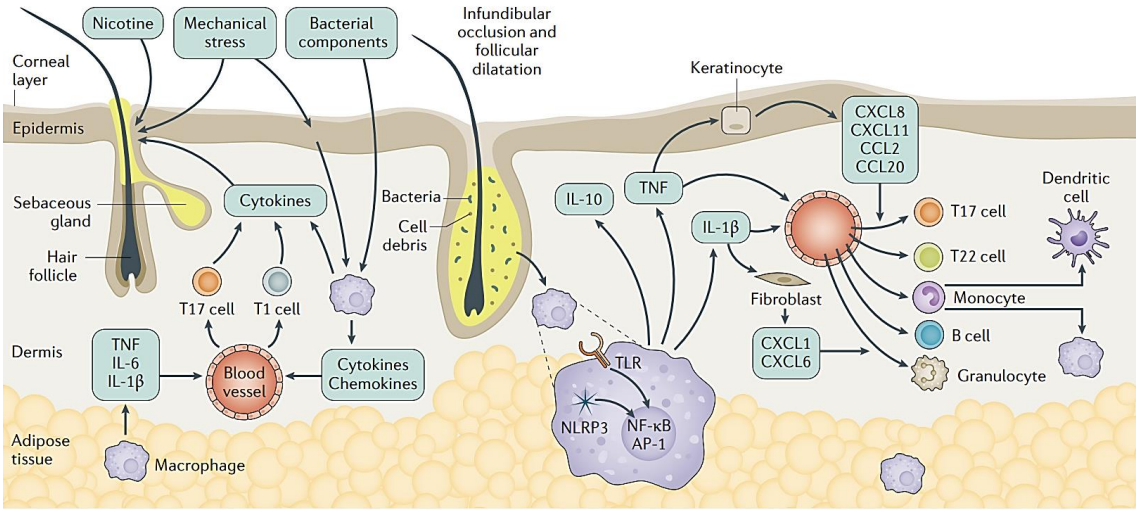

Figure 1.7 | Initial events in the immunopathogenesis of HS. The follicular occlusion and bacterial propagation are the stimuli required for the activation of macrophages via TLR. Consequently, IL-1β and TNF-α pro-inflammatory cytokines are secreted, leading to endothelial cell activation and chemokine production. As a result, several immune cells (e.g., neutrophils, monocytes, T and B cells) are recruited to the inflammatory site that will conduct immune responses towards a chronic inflammatory state.[61]

Following the recruitment of monocytes, these cells can either differentiate into macrophages or DCs. The latest can activate T lymphocytes and redirect their maturation into different subsets of T cells, based on the cytokines released by DCs (**Figure 1.8**). Therefore, IL-12 and IL-23 cytokines will favour Th1 and Th17 phenotypes, respectively. Th1 cells are commonly known to produce IFNγ that, in the context of HS, will simultaneously activate endothelial cells and induce the production of chemokines (e.g., CXCL10), resulting in the recruitment of immune cells.[62,63] Activated Th17 cells will release IL-17 to induce chemokines-mediated immune cell recruitment while generating antimicrobial peptides (AMPs). Moreover, high levels of IL-10 secreted by macrophages play an important role in HS onset, once this anti-inflammatory cytokine has the potential to decrease T cell activity, namely Th22 cells. Thus, the levels of both IL-22 and AMPs become compromised, as this cytokine is a better inducer of AMPs rather than IL-17.[64] Thus, the low levels of AMPs in HS skin are unable to control bacterial propagation, resulting in hair follicle dilatation. Eventually, this culminates in follicular epithelium rupture and release of bacterial content into the skin, contributing to a continuously exacerbated immune response. On the other hand, macrophages can detect these PAMPs and DAMPs via TLR, increasing the levels of TNF-α and IL-1β and promoting immune infiltration and the typical inflammatory nodules and abscesses of HS skin. Neutrophils can be recruited to the inflammation site, being the major contributors to pus formation and lipocalin 2 secretion (that further attracts more neutrophils). Furthermore, IL-1β induces the production of matrix metalloproteinases (MMPs) by fibroblast, which leads to tissue destruction.[61] Eventually, T lymphocytes will induce the activation of keratinocytes leading to a higher thickness of skin epidermis, commonly known as epidermal acanthosis. This, combined with the accumulation of pus and tissue destruction, will promote the formation of fistulas and sinus tracts typical of HS lesions.

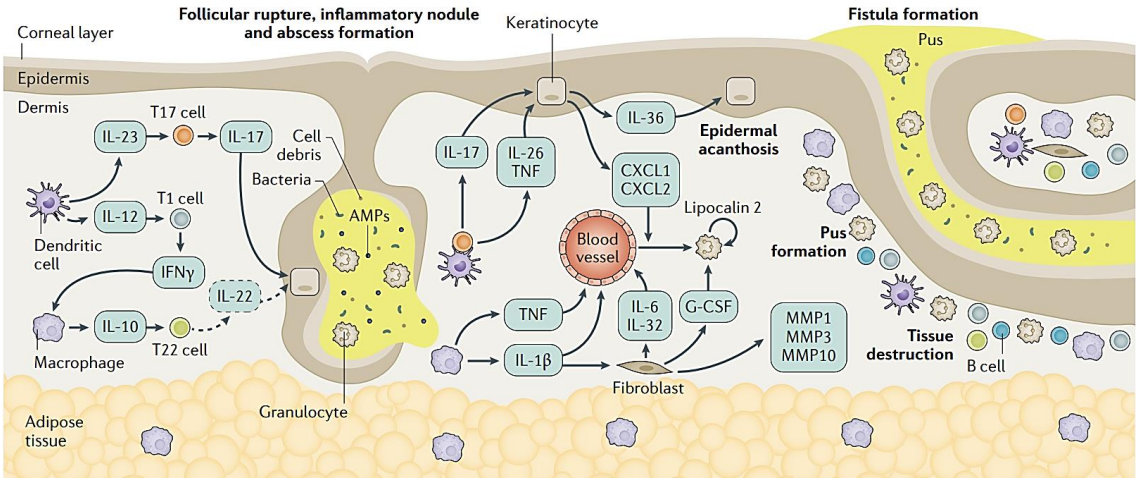

Figure 1.8 | Advanced mechanism of the immunopathogenesis of HS. Th1 and Th17 cells are important to produce IFNγ and IL-17 that potentiate the recruitment of immune cells to the inflammatory site. The propagation of bacteria will culminate in the follicular rupture and the subsequent activation of macrophages through TLR. As a result, tissue destruction is induced by activated fibroblasts, pus formation is ensured by neutrophils and activated keratinocytes lead to epidermal acanthosis. Finally, inflammatory nodules, abscesses, fistulas and sinus tract are generated as the typical morphological lesions of HS patients.[61]

1.2.3. Fibrocytes

Proposed by Bucala *et al.* [65] in 1994, the term fibrocytes was created to describe a novel immune population of hematopoietic-derived, fibroblast-like, spindle-shaped and adherent cells. Fibrocytes are present in the peripheral blood, unlike connective tissue-resident fibroblasts, and their recruitment to injury sites relates to their potential for subcutaneous wound healing, which ultimately may result in fibrosis and scar formation due to collagen production.

Afterwards, several studies were conducted to better understand the characteristics of this unique cell type, including the *In vitro* culture of human peripheral blood mononuclear cells (PBMCs) and the subsequent analysis of their extracellular markers. Unfortunately, the unequivocal identification of fibrocytes is very complex since no unique marker for these cells is available, requiring the combination of several strategies: immunolabeling (Identification of extracellular markers, commonly known as cluster of differentiation (CD)), labelling of circulation cells and transcriptional analysis.[66] Fibrocytes express both hematopoietic and stromal cell markers, including CD45 (leukocyte common antigen), CD34 (hematopoietic stem cell antigen), CD11b (myeloid integrin, adhesion molecules), CD13 (pan-myeloid antigen), CD86/CD80 (costimulatory markers), MHC II (antigen presentation) and collagen type I and II (extracellular matrix proteins). Additionally, they also express chemokines receptors (e.g., CC-chemokine receptor 2 (CCR2), CCR7 and CXCR4), important for the migration of fibrocytes towards inflammatory sites, and alpha-smooth muscle actin (αSMA), secreted by cultured fibrocytes and important for wound repair.[66,67]

Fibrocytes are a unique immune cell population that contributes to both innate and adaptive immune responses. Their role in innate immunity is associated with the production of antimicrobial factors to limit bacterial propagation at infection sites (e.g., myeloperoxidase involved in ROS synthesis, cathelicidin and defensins), and DNA-based extracellular traps.[68] Regarding the adaptive immune system, and similarly to other APCs, fibrocytes are capable of presenting antigens to naïve T cells (via MHC II and costimulatory molecules CD86 and CD80) and induce their activation.[69] Interestingly, a cluster of fibrocytes with antitumoral properties was described to infiltrate the tumours, increasing the efficacy of anti-PD-L1 immunotherapies while stimulating $CD8^+$ T cells within the tumour microenvironment, revealing the promising role of fibrocytes in immune-related diseases.[70]

Although fibrocytes have an important function in maintaining host homeostasis, either by the secretion of cytokines (e.g., IFNγ, IL-6, IL-8, TNF-α, IL-1β, IL-10), chemokines (e.g., CC-chemokine ligand 2 (CCL3), CCL3, CCL4, CXCL1, CXCL2 and CXCL8) or the processes mentioned above, they can also assume pro-inflammatory characteristics implicated with chronic inflammation and autoimmunity (**Figure 1.9**).[71,72]

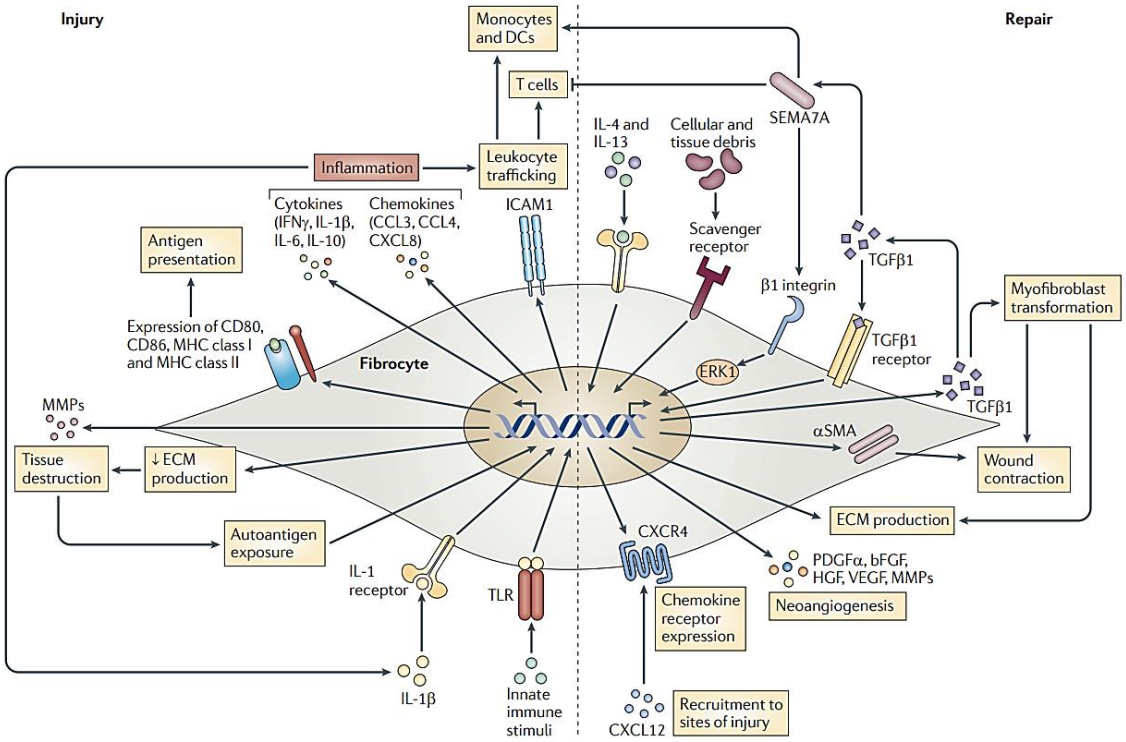

Figure 1.9 | The role of fibrocytes in autoimmunity. In an autoimmune scenario, whether triggered by a self-antigen exposure, injury, IL-1β or innate stimuli, fibrocytes assume a pro-inflammatory phenotype characterized by the production of pro-inflammatory cytokines (e.g., IFNγ, IL-6, IL-8, TNF-α, IL-1β, IL-10) and chemokines (e.g., CCL3, CCL4) that drive inflammation. Leukocyte recruitment to the inflammatory site is promoted by chemokines and intracellular adhesion molecule 1 (ICAM1). Fibrocytes will prime naïve T cells due to their antigen-presentation abilities via MHC I, MHC II, CD86 and CD80 expression. Finally, fibrocytes may induce tissue destruction by the secretion of MMPs.[71]

In fact, although the percentage of fibrocytes within the circulatory leukocytes is relatively low (approximately 5%), [65] several studies have demonstrated an increasing prevalence of fibrocytes in several chronic inflammatory diseases, including autoimmune disorders like RA [73], SSc [74], CD [75] and Graves' disease.[76] The increased number of fibrocytes may be related to the continuous activation of self-reactive $CD4^+$ T cells, a hallmark in autoimmunity, resulting in alarming disease severity and progression.[77] Consequently, some authors have focused on identifying the mechanisms that control fibrocyte outgrowth. Pilling *et al.* [78] have demonstrated that serum amyloid P (SAP) inhibits fibrocyte differentiation from $CD14^+$ monocytes, suggesting that low levels of SAP may contribute to chronic inflammatory disorders. Therefore, the use of SAP as a therapeutic agent in autoimmunity has been suggested to control the high levels of fibrocytes in these pathologies.[79]

Afterwards, fibrocytes present characteristics of both macrophages (pro-inflammatory phenotype) and fibroblasts (tissue-remodelling properties), presenting a clear role in disease initiation and progression to a chronic state.[80] Although fibrocytes constitute an attractive therapeutic target, further studies are required to better understand how these unique cells may interact with other immune cells to establish a chronic inflammatory state in IMIDs.[81]

1.3. Protein Glycosylation

Glycobiology is an expanding research field, aiming to study the structure, chemistry, biosynthesis and biological function of saccharides, commonly known as carbohydrates, glycans or sugar chains.[82] The glycocalyx represents the complex and diverse repertoire of glycans at the cell surface of all organisms, constituting a molecular interface between cells and the extracellular environment. Glycans harbour an enormous amount of information and are usually implicated in a wide range of cellular and biological processes in both homeostatic and pathological conditions. In fact, glycans are important for cell-cell interactions, cell adhesion, molecular trafficking and clearance, endocytosis, receptor activation, signal transduction and immune surveillance.[83] Additionally, in a pathological scenario, glycans are also involved in pathogen invasion, inflammatory reactions, autoimmunity and tumour metastasis.[84]

Glycosylation is a post-translational modification where glycoconjugates are enzymatically synthesized by the addition of glycans to other saccharides, through a glycosidic linkage, to either proteins or lipids. This process is conducted in the endoplasmic reticulum (ER) and Golgi apparatus, and it comprises a complex interplay of several enzymes (glycosyltransferases and glycosidases) and carbohydrates in a process controlled by the availability of activated substrate donors, enzyme activity, abundance and location.[84] The numerous glycan structural combinations result in a unique and diverse glycome of each cell and several classes of glycoproteins (*N*-linked glycans, *O*-linked glycans, glycosaminoglycans and glycosylphosphatidylinositol (GPI) anchors), and glycolipids (glycosphingolipids) can be found in nature (**Figure 1.10**).[85] Moreover, the *N*-linked glycans and *O*-linked glycans are the two main types of glycoproteins and include the addition of a carbohydrate to a nitrogen atom of asparagine (Asn) and to an oxygen atom of serine (Ser) or threonine (Thr), respectively.

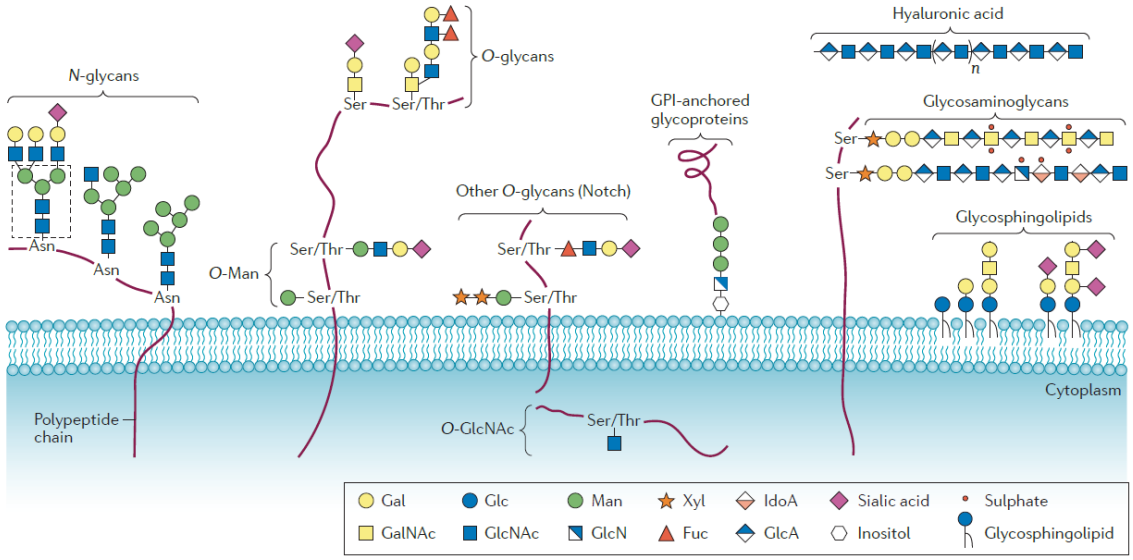

Figure 1.10 | Classes of glycoconjugates in mammals, with the respective nomenclature of saccharides. Glycoconjugates include glycoproteins (*N*-linked glycans, *O*-linked glycans, glycosaminoglycans and glycosylphosphatidylinositol (GPI) anchors) and glycolipids (glycosphingolipids).[85]

1.3.1. *N*-glycosylation

N-glycosylation is widely explored in eukaryotic species since glycoproteins with this post-translational modification account for approximately half of all human proteins.[86] As already mentioned, *N*-glycans are formed when a carbohydrate is added to a nitrogen atom of Asn, within a consensus sequence Asn-X-Ser/Thr, where X stands for any amino acid other than Proline (Pro). Although the great diversity of glycans, all eukaryotic *N*-glycans share a common core structure: Mannose(Man)α1,3(Manα1,6)Manβ1,4N-acetylglucosamine(GlcNAc)β1,4GlcNAcβ1-Asn-X-Ser/ Thr. *N*-glycosylation comprises three types of *N*-glycans (**Figure 1.11**): oligommanose or high-mannose (the glycan structure is only composed of Man residues), complex (the glycan structure possesses an extended core, initiated by GlcNAc residue) and hybrid glycans (the glycan structure is simultaneously composed by a Man extended Manα1,6 core arm and the GlcNAc extended Manα1,3 core arm).[87]

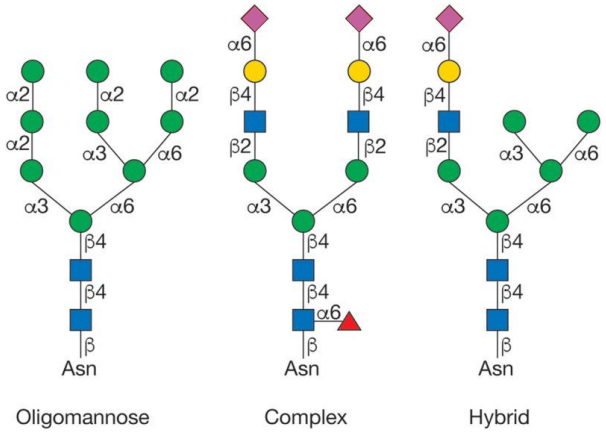

Figure 1.11 | Types of *N*-glycans. Within the consensus sequence Asn-X-Ser/Thr, there are three main types of *N*-glycans: oligommanose, complex and hybrid. *Adapted from* [87].

This process (**Figure 1.12**) starts in the ER membrane and lumen with the formation of a lipid-glycan precursor, where a glycan structure ($Glc_3Man_9GlcNAc_2$) is attached to dolichol phosphate (Dol-P), supported by the enzymatic activity of asparagine-linked glycosylation (ALG) and the presence of UDP-GlcNAc and GDP-Man activated substrate donors.[88] Subsequently, the lipid-glycan precursor is transferred to the side chain of an Asn residue (within the glycosylation site, Asn-X-Ser/Thr) by oligosaccharyltransferase (OST), while the protein is being translated in the ribosome. The following events include the sequential trimming of *N*-glycans by the combined action of glycosidases (α-Glc I-II) and mannosidases (ER α-Man), resulting in a Man_8GlcNA_2 structure (high-mannose *N*-glycans) that will continue its maturation in Golgi apparatus.[89] Importantly, during the trimming process, an alternative Man_7GlcNA_2 precursor can be synthesized and attached to an unfolded peptide, allowing its recognition by calnexin and calreticulin glycan-binding proteins (GBP, also called lectins) and the subsequent targeting for ER-associated degradation (ERAD), as a quality control mechanism.[90]

In *cis*-Golgi, the glycoprotein is further trimmed to Man_5GlcNA_2, due to the removal of α1,2Man residues by α-Man IA, IB and IC, followed by the addition of a β1,2GlcNAc residue by N-acetylglucosaminyltransferase I (GnT-I enzyme, encoded by *MGAT1* gene). From here, two alternative pathways into *medial*-Golgi are available: Gal-T or α-Man II (*MAN2A1* and *MAN2A2*) enzymatic action. The first one is important for the establishment of hybrid *N*-glycans, by the addition of β1,4 galactose (Gal), where the latest will promote the removal of α1,3Man and α1,6Man residues, resulting in the $GlcNAcMan_3GlcNAc_2$ structure that will further maturate into complex *N*-glycan. The GnT-II (*MGAT2* gene) acts in this instance to add a second β1,2GlcNAc residue, establishing the precursor for all biantennary and complex *N*-glycans. Additional branches, and tetra-antennary glycans, can be synthesized by the combined action of GnT-IV (*MGAT4*) and GnT-V (*MGAT5*) that catalyses the addition of a β1,4GlcNAc to core α1,3Man and β1,6GlcNAc to core α1,6Man, respectively. Furthermore, a bisecting GlcNAc residue can be added to the core β14Man in both complex and hybrid *N*-glycans, by GnT-III (*MGAT3*).[87,89]

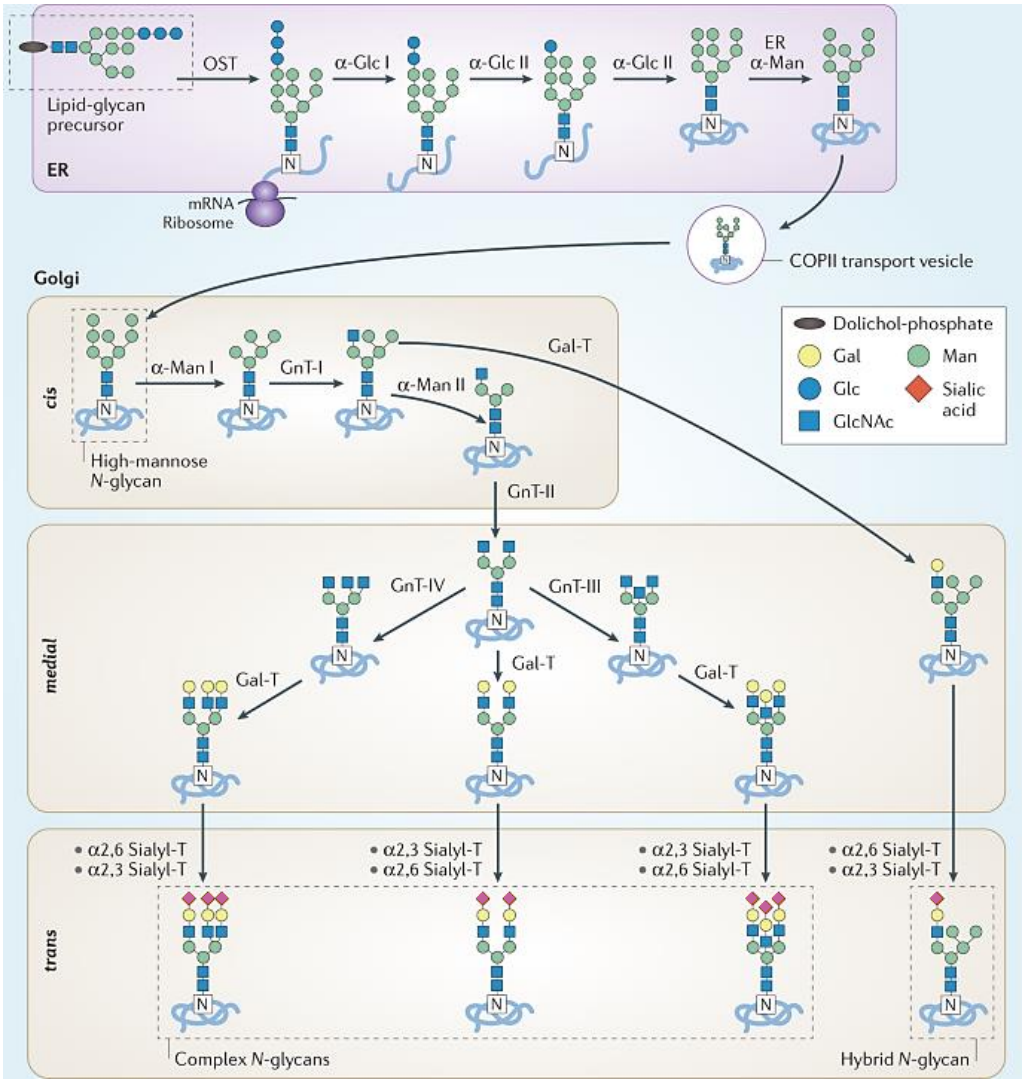

Figure 1.12 | Principal steps of the *N*-glycosylation pathway in ER and Golgi apparatus. This process is ensured by several enzymes (glycosyltransferases, glycosidases and mannosidases), that together, are responsible for the production of the high-mannose (mostly located in *cis*-Golgi), hybrid and complex *N*-glycans (where its maturation occurs in the *medial* and *trans*-Golgi). *Adapted from* .[89]

The final maturation of *N*-glycans takes place in the *trans*-Golgi, with the subsequent elongation of branches with a variety of carbohydrate structures, giving rise to the complex and diverse repertoire of glycans at the cell surface. These alterations may include: the addition of saccharides to the *N*-glycan core, namely the addition of α1,6 fucose (Fuc) residue to the core GlcNAc linked to Asp by *FUT8* encoded enzyme; the elongation of GlcNAc branches, followed by the addition of tandem repeats of Galβ1,4GlcNAc residues, commonly known as poly-*N*-acetyllactosamine (poly-LacNAc); the final addition of Fuc, Gal, GlcNAc and *N*-acetylneuraminic acid (NeuAc), also known as sialic acid, to the elongated branches of hybrid and complex *N*-glycans, important for the glycan recognition by lectins and antibodies.[87]

1.3.2. Glycoimmunology, the role of glycans in immune systems

Glycoimmunology is a research field focused on the role of glycans in the regulation of both innate and adaptive immunity, being important to pathogen recognition, homeostatic immune responses and inflammation. Glycans mediate a variety of cellular and molecular mechanisms, presenting both stimulatory and inhibitory immune properties that modulate the development of cancer and autoimmunity.[91] In fact, glycans are involved in leukocyte recruitment towards an infection site [92], the development of T (including the differentiation into either pro- or anti-inflammatory T cells subsets) and B cells, in which the released antibodies can assume either pro- or anti-inflammatory properties, depending on their glycosylation profile.[93] The immune regulation by glycans is highly dependent on their recognition by lectins (or GBP), which can decode information within the cellular glycome. These GBP have carbohydrate-recognition domains (CRDs) directly involved in the glycan binding, and several families of receptors are important for this recognition, including galectins, C-type lectins (CTLs) and siglecs.[94]

Galectins are soluble lectins capable of recognizing Galβ1,4GlcNAc residues (poly-LacNAc) and, unlike other GBPs, galectins can be located in both intra and extracellular space. Within the intracellular environment, galectins are involved in cell signalling, survival, phagocytosis and autophagy, whereas in the extracellular space, they can mediate cell-cell interactions, cell adhesion and activation, cytokine secretion, chemotaxis and cell fate.[95] Galectins are highly expressed by both innate and adaptive immune cells, including macrophages, DC, T and B lymphocytes, being responsible for regulating their immune activity. Demetriou *et al.* showed that galectin-3 inhibits T-cell activity by binding to TCR-complex glycans, increasing the activation threshold due to TCR clustering impairment.[96] Additionally, Dias *et al.*, demonstrated that a deficiency in complex *N*-glycans from $CD4^+$ T cells in active UC patients is related to a hyperactive immune response, concluding that the abolishment of complex glycans within TCR is associated with an increased susceptibility to develop autoimmunity.[97] Moreover, galectin-9 is also described to regulate the activation threshold of B lymphocytes, since this GBP can simultaneously bind to poly-LacNAc residues in CD22, CD45 and BCR from naïve B cells, suppressing BCR signalling, B cell activation and differentiation into antibody-secreting plasma cells.[98]

CTLs are a large family of calcium-dependent glycan-binding proteins, consisting of 17 subgroups according to their phylogeny and domain organization. In mammalian species, these GBPs can be either secreted or localized at the immune cell surface as transmembrane C-type lectin receptors (CLRs), which modulate cellular development, homeostasis and immune responses. Furthermore, CTLs can be classified into two main groups based on a conserved amino acid sequence within the CRDs: EPN (Glu-Pro-Asn) and QPD (Gln-Pro-Asp), which relates to their specificity to mannose-type and galactose-type saccharides, respectively.[99] These lectins are highly abundant in APCs (e.g., macrophages and DC), in NK and endothelial cells, to drive leukocyte recruitment. Some of these lectins include DC-SIGN, L-SIGN, mannose receptor (MR), macrophage galactose-specific lectin (MGL) and langerin, and they present specific binding properties towards high-mannose, Fuc and GalNAc residues.[94,100] Oligomannosidic epitopes are a common feature among several pathogens and simpler organisms, therefore, DC-SIGN, langerin and MR in APCs are important in the context of infection to recognize and internalize the foreign antigen and drive the activation of naïve T cells into Th1 or Th17 cells via MHC I and II, important to establish an antimicrobial immune response.[94,100] Dectin-1 is another CTL involved in the uptake of pathogens and in inducing an immune response due to its great affinity towards β-glucan, an important glycan component of fungi. Given that, many authors have suggested a similar role between CTLs and PRRs. Additionally, CTLs can recognize self and non-self antigens, which is important to maintain immune tolerance to prevent autoimmune disorders.[99]

Siglecs are a family of membrane proteins with high specificity to sialylated structures on both *N*- and *O*- glycans while presenting Ig-like binding domains. Siglecs can be divided into two groups, including the CD33-related siglecs and another one composed of sialoadhesin (siglec-1), MAG (siglec-4) and CD22 (siglec-2).[94] These lectins are important regulators of immune responses, specifically by the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in CD22 and CD33-related siglecs that suppress cell signalling. Additionally, siglecs are mostly expressed by neutrophils, monocytes, macrophages (siglec-1), DC, NK and B cells (siglecs-2, which induces the inhibition of BCR, modulating B-cell function, activation threshold and survival), while its expression on T cells is almost negligible. Pathogens may also express these GBPs, revealing their importance in pathogen recognition and communication with immune cells.[101]

1.3.3. Glycans in autoimmunity

Glycans are crucial for the identity of a cell, being expressed by both humans and pathogens. In fact, glycosylation complexity is an evolutionary mechanism, where microorganisms like bacteria, fungi and viruses display a more simplistic glycome (enriched in mannan structures and highly mannosidic *N*-glycans), in contrast to higher organisms like humans (**Figure 1.13**). Consequently, the different glycosylation patterns allow the immune system to discriminate self from nonself antigens (immune tolerance) by glycan recognition through GBP, triggering a pro-inflammatory immune response to clear the pathogen.[102,103]

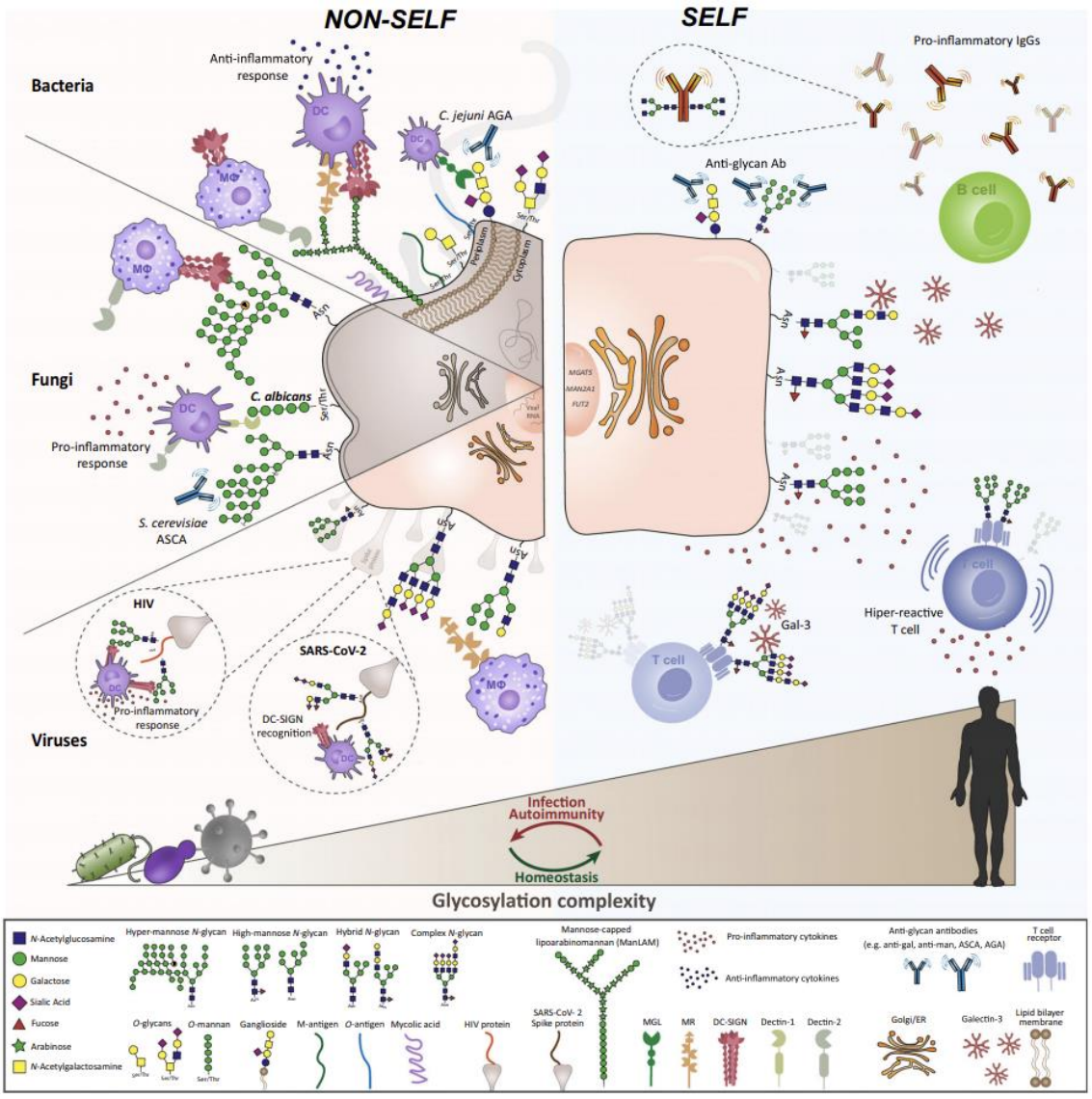

Figure 1.13 | Glycans are important for the identity of a cell or organism, allowing the discrimination between self and nonself antigens (immune tolerance). Alterations in host glycosylation may trigger the development of autoimmunity, through a process called "Glycan mimicry".[102]

Alterations in host glycosylation, to less complex microbial-associated glycans, can be recognized by GBP that will further activate pro-inflammatory responses against the host. This mechanism is denominated "Glycan mimicry" which leads to the loss of immune tolerance and, consequently, to the development of autoimmune disorders.[102] Alves *et al.* have demonstrated that SLE patients display an abnormal exposure of mannose-enriched glycans and their recognition by DC-SIGN-expressing γδ T cells triggers a pathogenic autoimmune response, mediated by IL-17a pro-inflammatory cytokine.[104,105] Additionally, increased levels of autoreactive anti-glycans antibodies have been associated with IMIDs, namely in CD patients, where the high titters of ASCA IgGs can be used as a diagnostic and predictive biomarker for this disease.[32,106] Afterwards, positivity for ASCA may indicate a systemic response to the oligomannosidic epitopes of microorganism, since ASCA recognize mannan structures from *S. cerevisiae*, being an important driver in immune tolerance impairment and, consequently, autoimmunity.

1.4. Aim of the project

The goal of this work is to determine whether the exposure of abnormal, yet similar, glycosylation profile in skin and gut from HS and CD patients, respectively, may stimulate the common production of anti-glycan antibodies (namely ASCA IgG) triggering the activation of a pro-inflammatory response in skin and colon, as a potential novel gut-skin axis in HS and CD.

In order to achieve this, we aim to:

1. Characterize the glycome profile of skin and gut epithelium using human clinical samples in two different approaches (*in situ* and at the transcriptional level);

2. Evaluate the impact of the exposure of abnormal glycoantigens in IgG recognition associated with pro-inflammatory responses;

3. Evaluate the potential of serological anti-glycan antibodies in the establishment of a gut-skin axis in HS and CD patients;

4. Explore the role of fibrocytes in the immunopathogenesis of HS;

5. To evaluate the presence of a gut-skin axis in a glycoengineered mouse model susceptible to colitis.

2. MATERIALS & METHODS

2.1. Patients' Cohort

This study includes formalin-fixed paraffin-embedded (FFPE) samples, patient's isolated IgGs and snap-frozen tissue, including the healthy controls (HC), CD and HS patients. Regarding HC, the biological samples were kindly provided by Centro Hospitalar Universitário (CHU) de São João, Porto, including FFPE samples (skin n=7, colon n=4), FFPE tissue for RNA extraction (skin n=6), serological antibodies (n=5) and snap frozen tissue for protein/RNA extraction (colon n=3). For the CD patients, this study includes FFPE samples (n=4), serological antibodies (n=5) and snap-frozen tissue for protein/RNA extraction (colon n=6). Lastly, biological samples from HS patients were kindly provided by NYU Langone Health Institute, USA, and include FFPE samples (n=6), snap frozen tissue for RNA extraction (skin n=10) and patient's isolated antibodies (n=15).

2.2. RNA Sequencing Data from HS Patients

RNA seq data results from a collaboration with Drª. Catherine Pei-Ju Lu and Drª. Joy Barrett from NYU Langone Health Institute in New York, USA. This was performed on skin tissue from HS patients, normalized to the skin from healthy controls (HC), analysing both immune ($CD45^+$ cells) and non-immune compartments ($CD45^-$ cells).

2.3. Lectin histochemistry and Immunohistochemistry

Histochemistry is a powerful microscopy-based approach that combines biochemistry and histology techniques, involving the use of staining agents to analyse the chemical composition of a tissue. In this case, histochemistry was employed to characterize the glycan profile of FFPE tissue samples from HC (skin n=7; colon n=4), HS (n=6), and CD (n=4) patients.

Firstly, samples were deparaffinized in xylene, rehydrated with successive lower concentrations of ethanol (from 100% to 70%) and washed with water. Following endogenous peroxidase activity blockage (3% H_2O_2 in methanol) for 10 minutes, sections were blocked with bovine serum albumin (BSA, Sigma-Aldrich®) 10% in phosphate-buffered saline (PBS) for 30 minutes RT, to minimize non-specific binding interactions. Then, sections were stained with biotinylated lectins in PBS for 1 hour at RT: *Galanthus Nivalis Agglutinin* (GNA, 1:50 and 1:300 to colonic and skin tissue, respectively), which recognizes terminal α1,3Man residues (B-1245, Vector Laboratories), and *Sambucus Nigra Lectin* (SNA, 1:1500 in skin tissue), that recognizes the α2,6-linked sialic acid (B-1305, Vector Laboratories). After washing with PBS, sections were incubated with Vectastain ABC kit (Vectastain® ABC Kit, Vector Laboratories) for 30 minutes, 1:100 in PBS and RT to detect the avidin-biotin-peroxidase complex (ABC). Next, colour was

developed after incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB Quanto Detection System, Epredia™) for 5-8 minutes in the dark, due to the enzymatic oxidation of DAB by ABC complex. Lastly, all sections were counterstained with haematoxylin, dehydrated with successive higher concentrations of ethanol (from 90% to 100%) and preserved in the mounting medium (Entellan® new, Sigma-Aldrich®).

Regarding Mannitou, the immunohistochemistry (IHQ) was performed with a horseradish peroxidase (HRP) polymer detection kit (UltraVision™ Quanto Detection System HRP, Epredia™), following the manufacturer's procedures. After deparaffinization and rehydration, endogenous peroxidase was blocked with “UltraVision Hydrogen Peroxide” solution for 10 minutes, followed by “UltraVision Protein Block” for 5 minutes. Next, sections were stained with Mannitou, a monoclonal antibody that recognizes *S. cerevisiae*-like glycans and ASCA epitopes, 1:20 in PBS, ON at 4 ºC. On the following day, and after washing with PBST (PBS 0.05%Tween® 20, Sigma-Aldrich®), sections were incubated with “Primary Antibody Amplifier Quanto” solution for 10 minutes, followed by “HRP polymer Quanto” for 10 minutes in the dark. Lastly, sections were incubated with DAB, counterstained with haematoxylin, dehydrated and preserved in Entellan.

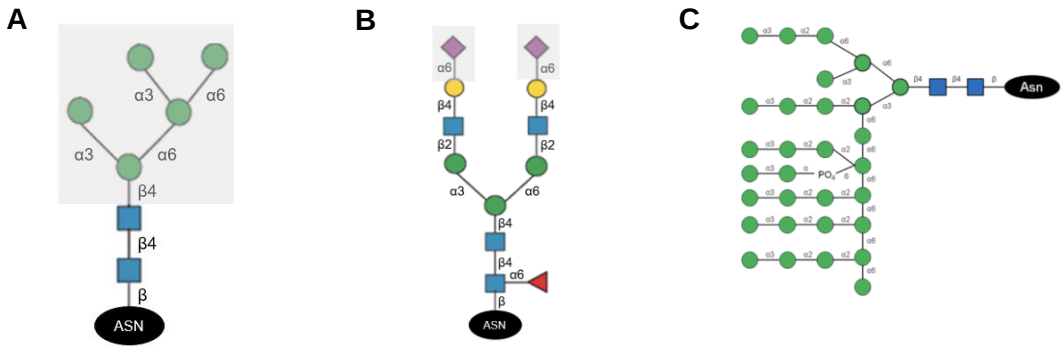

Figure 2.1 | General glycan binding motifs. (A) GNA lectin recognizes terminal α1,3Man and α1,6Man residues. **(B)** SNA lectin recognizes α2,6-linked sialic acid. **(C)** Mannitou-like-binding site, mannan structures typically from *S. cerevisiae*. Figures A and B are adapted from [107], and Figure C from [108].

Afterwards, lectin reactivity was quantitatively and qualitatively analysed. Quantitative analysis was conducted in ImageJ v8 software, and it comprises the conversion of intensity numbers into optical density (OD) of deconvoluted images using the following equation:

$$OD = \log\left(\frac{Maximum\ intensity}{Mean\ intensity}\right), where\ the\ maximum\ intensity = 255, for\ 8-bit\ images$$

The qualitative analysis was conducted by three independent observers, considering three levels of reactivity: Low (0-25%), Medium (25-75%), and High (> 75%).

2.4. RNA extraction from FFPE and snap-frozen tissue

The RNA from HC was isolated from FFPE tissue samples, whether in HS and CD patients was extracted from snap-frozen tissue.

Regarding the FFPE samples, total RNA was extracted with Recover All™ Total Nucleic Acid Isolation Kit (Invitrogen), according to the manufacturer's procedures. Briefly, sections were deparaffinized with 100% xylene for 5 min at 50 ºC, centrifuged at maximum speed to remove xylene, washed twice with 100% ethanol and then the pellet was air dried for 1 hour at RT. Next, samples were incubated with digestion buffer and protease at 50 ºC for 15 minutes, followed by another 15 minutes at 80 ºC. Then, samples were passed through a filter cartridge column that retains the RNA, upon the addition of an isolation additive solution with 100% ethanol. The column was washed several times with wash 1 and wash 2/3 solutions, followed by centrifugation at 10 000 *xg* for 30 seconds. To remove contaminating genomic DNA, DNase mix was applied to the filter cartridge for 30 minutes at RT, followed by a washing step with wash 1 and wash 2/3 solutions. Finally, RNA was recovered with the elution solution and stored at – 80 ºC.

For the HS cohort, samples were incubated with TRIzol™ Reagent (Sigma-Aldrich®), followed by tissue dissociation with TissueRuptor® II (Qiagen), and the addition of glycogen (Roche®) for 5 minutes at RT. Next, chloroform (Sigma-Aldrich®) was added for 5 minutes at RT to separate the RNA from the remaining components, and then samples were centrifuged for 10 minutes at 18 000 *xg* and 4 ºC. The upper phase (aqueous phase) was transferred to a new clean tube, followed by the addition of isopropanol (Sigma-Aldrich®) and then the samples were stored ON at – 20 ºC, so that RNA could precipitate. On the following day, samples were centrifuged at 4 ºC for 30 minutes at 18 000 *xg* and the pellet was washed with ethanol 75%, followed by another centrifugation at 7500 *xg* for 15 minutes. Finally, the pellet was left air-dried, dissolved in UltraPure™ DNAse/RNAse-free water (UP H_2O, Invitrogen) and stored at – 80 ºC.

2.5. Real-time qPCR

Quantitative real-time polymerase chain reaction (RT-qPCR) is a molecular biology approach that enables the quantification of DNA or RNA sequences during amplification. In this case, RNA concentration was firstly measured with NanoDrop™ One microvolume UV-Vis Spectrophotometer (Thermo Scientific™) and 60 ng of RNA was reversibly transcribed into complementary DNA (cDNA). Briefly, samples were diluted in UP H_2O, followed by the addition of random primers (stock concentration 0.1 µg/mL, Invitrogen) and deoxynucleotides triphosphates (dNTPs, 10 mM, Thermo Scientific™). Then, samples were incubated in T100™ Thermocycler (Bio-Rad laboratories) for 65 ºC (5 minutes) and 4 ºC (1 minute), to allow the annealing of primers to the template RNA. Next, a reaction mix containing 5x PCR Buffer (Invitrogen), dithiothreitol (DTT, New England BioLabs), RNase OUT (Invitrogen) and SuperScript IV™ reverse transcriptase (Invitrogen) enzymes were added to the samples. Finally, cDNA was

synthesized through a second temperature cycle consisting of 23 ºC (10 minutes), 50 ºC (10 minutes), 80 ºC (10 minutes) and 4 ºC (∞), that ensures primers annealing and the elongation of DNA sequence.

The RT-qPCR allows the cDNA amplification detection in real-time through the generation of fluorescence, by using either specific probes (TaqMan) or primers (SYBR Green) for the gene of interest. TaqMan™ probes are equipped with a fluorescent reporter that is released when the sequence of interest is amplified, whereas the SYBR green method involves the incorporation of a fluorescent dye into the dsDNA of the gene of interest, while it is being synthesized.[109] In this case, the RT-qPCR was performed in a MicroAmp™ Fast Optical 96-well reaction plate, where each well contains the cDNA (or water in the case of negative control) and the reaction mix, that can be either TaqMan™ (Master Mix II (Applied Biosystems), TaqMan™ probe for target gene, UP H_2O) or SYBR Green (SYBR® Green I Master mix (Roche), forward and reverse primers (Invitrogen) mix of the target gene, ROX reference dye (Sigma-Aldrich®) and UP H_2O).

Afterwards, the target glycogenes include:

- *MGAT1* (α1,3-Mannosyl glycoprotein 2-β-*N*-acetylglucosaminyltransferase, TaqMan™, Hs00159121_m1);
- *MAN2A1* (Mannosidase α class 2A member 1, TaqMan™, Hs01123597_m1);
- *MGAT5* (α1,6-Mannosylglycoprotein 6-β-*N*-acetylglucosaminyltransferase, TaqMan™, Hs00159136_m1);
- *POMT1* (Protein *O*-Mannosyltransferase 1, TaqMan™, Hs01059558_m1);
- *ST6Gal1* (ST6 β-Galactoside α2,6-Sialyltransferase 1, SYBR Green primers sequence in **Appendix 1**);
- *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase, TaqMan™, Hs02786624_m1, SYBR Green primers sequence in **Appendix 1**);
- *HPRT* (Hypoxanthine phosphoribosyltransferase 1, TaqMan™, Hs02800695_m1).

RT-qPCR data was acquired with 7500 Fast Real-Time PCR software (Applied Biosystems), following a holding stage of 50 ºC (20 seconds) and 95 ºC (10 minutes) and an amplification cycle of 95 ºC (15 seconds) and 60 ºC (1 minute) for 45 cycles. Finally, mRNA expression of target genes (ΔCt) was normalized to the housekeeping gene (*GADPH* or *HPRT*), and the subsequent RQ ($2^{-\Delta Ct}$) values were calculated.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) was performed to understand the affinity and specificity of total IgGs from HS and CD patients towards several glycoantigens: OVA-Man_9 and OVA-diGlcNAc. In this case, Man_9 and diGlcNAc structures were previously functionalized to a primary amine and then conjugated to ovalbumin (OVA).

The ELISA assay was performed in a 384-well plate and the first step comprised the coating of the plate ON at 4 ºC with increasing concentrations of glycoantigens in carbonate buffer (pH 9.6): 0, 10, 15, 25, 50, 100, 150 µg/mL. On the following day, the plate was incubated with blocking buffer (PBSA, PBS 2%BSA) for 6h at 4 ºC, followed by incubation with the patient's total IgGs (1:300) in dilution buffer (PBT, PBS 1%BSA 0.05%Tween) ON at 4 ºC. Importantly, the patient's total IgGs result from a pool of 8 and 5 samples, from HS and CD patients, respectively. On the third day, the plate was washed three times with PBT and incubated with anti-human IgG HRP-conjugated secondary antibody (A80-219p, Bethyl) in PBT (1:5000) for 1 hour at RT. After a washing step, tetramethylbenzidine (TMB, Invitrogen) substrate solution was added for 15 minutes, followed by sulfuric acid (2N H_2SO_4) to stop the reaction and, finally, the plate absorbance at 450 nm was measured in Synergy Mx Plate reader (Biotek).

Afterwards, another ELISA assay was conducted to characterize the levels of anti-Man_9 and anti-diGlcNAc antibodies in several IMIDs, including CD, HS, SLE, IIM and Sj. The procedure is similar to that described above, although the coating of the plate was done with 10 µg/mL of glycoantigen, and the HRP-conjugated secondary antibody was used in a dilution of 1:10 000.

2.7. Western blot assay

Western blot is a popular analytical technique that detects specific proteins in a sample, requiring protein separation according to their molecular mass and the subsequent recognition by a specific antibody. In this case, a Western blot assay was performed with colonic total cell lysate (TCL) from HC (n=2) and CD (n=3) and antibodies from HC and HS patients.

Firstly, the colonic protein lysate was obtained from HC and CD patients' snap-frozen tissue. Briefly, the colonic tissue was incubated with extraction buffer, composed of radioimmunoprecipitation assay buffer (RIPA), cOmplete (protease inhibitor cocktail, Roche®), phenylmethylsulphonyl fluoride (PMFS, protease inhibitor, Sigma-Aldrich®) and sodium orthovanadate (Na_3NO_4, tyrosine phosphatase inhibitor, Sigma-Aldrich®), followed by tissue dissociation with TissueRuptor® II (Qiagen). After a 15-minute incubation on ice, the samples were centrifuged at maximum speed (20 800 *xg*) for 10 minutes at 4 ºC, to separate the TCL from cellular debris. All the procedures must be carried out on ice to maintain sample integrity and prevent protein degradation and enzymatic reactions.

Moreover, the protein was quantified with the *DC* protein assay commercial kit (Bio-Rad Laboratories), following the manufacturer's procedures. This quantification method requires a calibration curve with BSA standard solutions and the protein lysate, diluted with Millipore-filtered (MilliQ) water, if necessary, to match the concentration intervals imposed by the calibration curve. Samples were incubated with reagents A and B for 15 minutes, revealing a bluish colour, the absorbance at 750 nm was measured in the Biotek MQX200 uQuant Microplate reader, and the concentration was calculated with the calibration curve.

Then, proteins were separated by Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE), which is based on the migration of negatively charged proteins towards the anode according to their molecular mass, by application of a constant electric field. In this case, samples were prepared with 1 µg of protein lysate (HC and CD patients), loading buffer (Blue Protein Loading Dye, 3x concentrate, New England BioLabs) and DTT (New England BioLabs), followed by denaturation at 95 ºC for 5 minutes. The electrophoretic separation was performed in two 10%/4% gels, with a constant voltage of 125 V, and the Dual Color (Bio-Rad Laboratories) was used as a protein marker to estimate protein molecular mass.

Following electrophoresis, the proteins were Wet-tank transferred to a nitrocellulose membrane (Amersham™, GE Healthcare) through a transfer stack, assembled in the following order from the cathode (-) to anode (+): sponge, three sheets of filter paper soaked in 1x transfer buffer (Bio-Rad Laboratories), gel, membrane, three sheets of filter paper soaked in 1x transfer buffer, sponge. The assembling of the transfer stack must be performed with precaution, to guarantee that the membrane is correctly hydrated and to avoid air bubbles that impair the transfer process. The transfer process is carried out at 60 V for 90 minutes and, to confirm the effectiveness of this process, the membrane was incubated with ponceau solution (Sigma-Aldrich®).

Afterwards, the membrane was subjected to blocking solution (1x Tris-buffered saline solution (TBS) with 5%BSA) under agitation for 2 hours at RT, to prevent non-specific binding of antibodies, followed by incubation with IgGs from HC (n=1) and HS patient (n=1) in TBS 1%BSA (1:1000), ON at 4 ºC under gentle agitation. On the following day, the membrane was washed two times with TBS 0.05%Tween (TBST) and incubated with Goat anti-human IgG HRP conjugated-secondary antibody (ab6858, Abcam) in TBST (1:10 000) for 45 minutes at RT. Then, the membrane was revealed in a dark room after incubation with ECL (Cytiva, Amersham™) for 2 minutes.

Finally, the membrane was incubated with vinculin as a loading control to normalize the results. Briefly, the membrane was washed with stripping solution (NZYtec), following the manufacturer's procedures and washed 3 times with TBST. Then, the membrane was blocked with 5% non-fat dry milk in TBST for 1 hour and incubated with anti-vinculin mouse primary antibody (V284 clone, Merck) in 1% non-fat dry milk in TBST (1:500) for 1 hour at RT. Followed by a washing step with TBST, the membrane was incubated with ECL™ anti-mouse IgG HRP

conjugated-secondary antibody (GE Healthcare) in 1% non-fat dry milk in TBST (1:2000) for 1 hour at RT. Lastly, the membrane was revealed in a dark room after incubation with ECL for 2 minutes, and the reactivity quantification was evaluated in ImageLab software.

2.8. Characterization of fibrocytes from Human PBMCs

The *In vitro* culture of fibrocytes required the isolation of human PBMCs from healthy donors' buffy coats, provided by CHU de São João, in a density-gradient centrifugation that allows the separation of cells according to their density (**Figure 2.2**).

Thus, 10 mL of blood was diluted in PBS and then carefully added to LymphoPrep™ (Stemcell™ Technologies), to avoid the breaking of the two phases and prevent the mixing of both components, in a ratio of 2:1. After a centrifugation step at 900 *xg*, for 30 minutes at RT, with no brake and no acceleration, the PBMCs were collected and washed two times with PBS, followed by centrifugation at 300 *xg* for 5 minutes at RT. Next, the pellet was resuspended and incubated with 1x Ammonium-chloride-potassium (ACK) for 5 minutes at RT, to lyse red blood cells (RBCs), centrifuged at 300 *xg* for 5 minutes at RT and, finally, the cells were counted in a hemocytometer (KOSA Plastics).

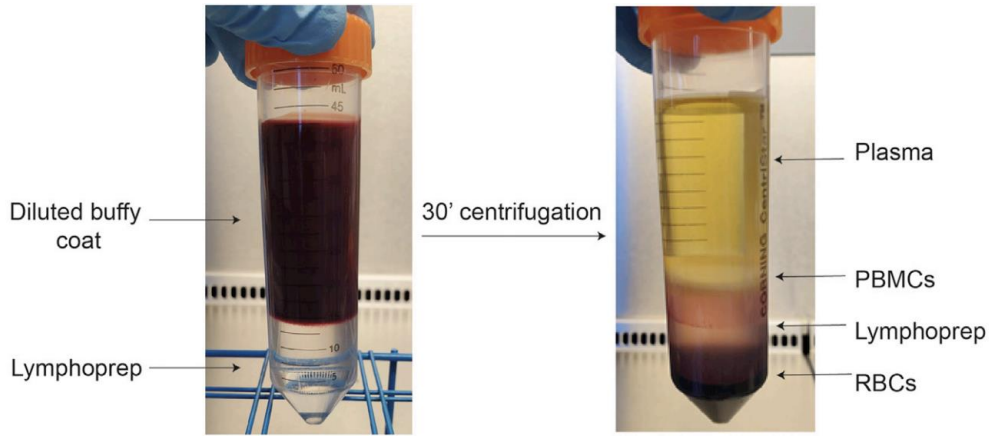

Figure 2.2 | PBMCs isolation in density-gradient centrifugation by Lymphoprep. After centrifugation, the blood cells are separated according to their density in RBCs, lymphoprep, PBMCs and plasma, where the PBMCs can be recovered by gentle aspiration.[110]

PBMCs were cultured in fibronectin (F2006, Merck) coated 6 well plates with 10 x 10^6 PBMCs per well, diluted in 5 mL of DMEM supplemented with 10% fetal bovine serum (FBS, VWR) and 1% Penicillin Streptomycin (P/S, Gibco™). After 3 days of continuous culture, nonadherent cells were removed by gentle aspiration (**Figure 2.3.A**) and fibrocytes were collected on day 10 with cold 0.05% Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich®), being recognized as adherent and spindle-shaped cells (**Figure 2.3.B**).

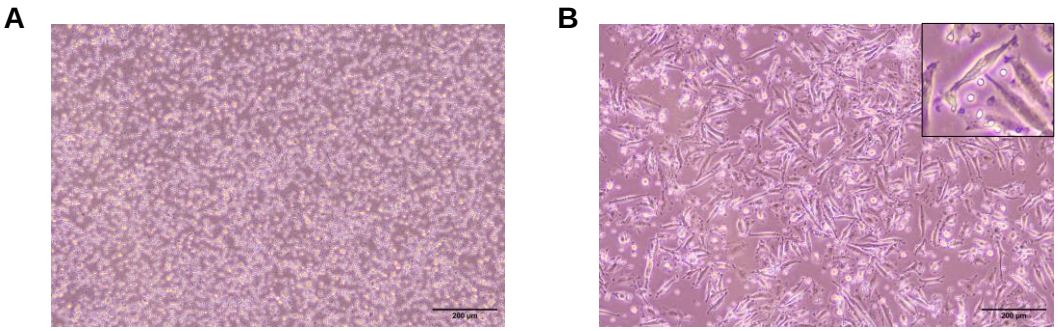

Figure 2.3 | Morphology of fibrocytes at day 3 (A) and day 10 (B) of *In vitro* continuous culture from human PBMCs. Ampliation 10x on the microscope.

Afterwards, fibrocytes (50 000 cells/well) were incubated with glycoantigens OVA-Man_9 and OVA-diGlcNAc (10 µg/mL) in DMEM 10%FBS 1%P/S, for 72h at 37 ºC in a 96-well flat plate. After 3 days, the supernatants were stored at – 80 ºC and the fibrocytes were analysed by Fluorescence-activated cell sorting (FACS). FACS is a powerful fluorescent-based technique, that allows the identification and quantification of cells, based on their physical and chemical characteristics (e.g., cell surface or intracellular markers), that are usually recognized by fluorochrome-conjugated antibodies. The cytometer is equipped with a variety of lasers, filters and detectors that analyse the emitted light of a fluorochrome, making it possible to use different fluorochromes that encounter the cytometer specificities. Therefore, this technique enables the simultaneous analysis of different cell populations within a sample, based on their immunophenotyping labelled by fluorochrome-conjugated antibodies.

With this in mind, fibrocytes were stained for Fixable viability dye (FVD, 1:2000), IgM (1:100), CD3 (1:100), CD14 (1:100), CD45 (1:200), CD34 (1:100), αSMA (1:100), CD86 (1:100), MHC II (1:200) and MRC2 (1:100) for 30 minutes at 4 ºC in the dark. After a washing step with 2% FACS buffer (PBS 2%FBS), cells were incubated with Donkey α-rabbit IgG (1:100) for 20 minutes at 4 ºC in the dark, to detect the presence of MRC2. Then, the cells were washed with 2% FACS and fixed with 2% paraformaldehyde (PFA) in PBS for 10 minutes at 4 ºC in the dark. Importantly, unstained and FVD controls were employed to better discriminate living and dead cells, during both acquisition and analysis processes. Additional information about the fluorochrome-conjugated antibodies is available in **Table 2.1**. Data acquisition was done in FACS Canto v.2 flow cytometer (BDBioscience), with FACSDiva software (BDBioscience) and files were analysed in FlowJo Software v.10.

Table 2.1 | Antibodies used in flow cytometry for fibrocytes characterization, their reactivity, clone, fluorochrome and supplier. APC (allophycocyanin), PB (pacific blue), PE (phycoerythrin), Cy (cyanine).

Reactivity	Antibodies	Clone	Fluorochrome	Supplier
Human/Mouse	FVD	-	APC-efluor 780	eBioscience
Human	IgM	SA-DA4	eFluor450	eBioscience
Human	CD3	OKT3	PB	BioLegend
Human	CD14	63D3	Brilliant Violet 421	BioLegend
Human	CD45	HI30	Brilliant Violet 510	BioLegend
Human	CD34	561	APC	BioLegend
Human, Mouse, Rat	αSMA	1A4	Alexa Fluor 488	eBioscience
Human	CD86	IT2.2	Pe-Cy5	Invitrogen
Human	MHC II	L243	PeCy7	eBioscience
Human, Mouse	MRC2	-	-	Novus
Donkey	α-rabbit IgG	-	PE	BioLegend

Afterwards, the supernatants were further evaluated for the presence of cytokines (IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70) using the Human Inflammatory Cytokine Cytometric Bead Array (CBA, BDBioscience) commercial kit, following the manufacturer's procedures. Briefly, this kit allows the simultaneous identification and quantification of cytokines by flow cytometer using specific APC-conjugated beads, with different intensities of PE fluorochrome.

2.9. Characterization of VT1 aging mouse model

The VT1 mice model is a conditional knock-out (KO) of the *Mgat1* gene in villin-positive cells, mainly expressed in the small intestine and colon, resulting in the accumulation of high mannose *N*-glycans at the surface of gut epithelia. We decided to use this animal model to better characterize a gut-skin axis, and to see if the abnormal glycoprofile in the colon would induce alterations in the skin (e.g., inflammation).

With this in mind, serological levels of ASCA-IgGs were quantified in VT1 wild-type (WT, n=4) and KO (n=5) aging mice with 60-68 weeks. Thus, blood was collected from the tail of these mice, followed by centrifugation at 775 *xg* for 10 minutes at RT and the collection of supernatants (serum). The quantification of ASCA levels was performed by QUANTA Lite ASCA (*S.cerevisiae*) IgG ELISA commercial KIT (Werfen), following the manufacturer's procedures. Briefly, the serum was added to the anti-ASCA-IgGs coated plates from the kit, which allowed a correct identification and quantification of these antibodies, followed by an incubation step for 30 minutes at RT. Then, the plate was washed 3 times with washing buffer, followed by 30 minutes (RT) incubation with anti-mouse IgG HRP-conjugated secondary antibody (A90-516p, Bethyl) in UP H_2O (1:10 000). After a washing step, TMB solution was added for 30 minutes at RT followed by HRP stop solution and, finally, the absorbance at 450 nm was measured in Synergy Mx Plate reader (BioTek).

Importantly, this test also includes a positive and negative control from the kit, to validate the results.

Afterwards, WT (n=6) and KO (n=6) VT1 aging mice were sacrificed by CO_2 inhalation, followed by skin collection for histopathological and FACS characterization. For the histopathological analysis, FFPE skin samples were sliced with a 4 µm thickness in the microtome (Microm HM335E), mounted in microscope slides (Avantor, VWR) and then stained for hematoxylin and eosin. Briefly, samples were deparaffinized and rehydrated, as previously described, followed by hematoxylin (Hematoxylin 7211, Epredia™) nucleic acid staining for 5 minutes. Then, samples were washed with water, embedded in ammoniacal water for 1 minute and then washed again for 5 minutes. Next, the samples were incubated with ethanol 95% and the cytoplasmatic proteins were stained with eosin (Eosin-Y, Epredia™) for 3 minutes. Finally, samples were washed with ethanol 95%, dehydrated and preserved in Entellan.

Then, skin single-cell suspension was obtained, as described by Sakamoto *et al.* [111], to perform FACS analysis. With this in mind, the skin was minced into small pieces and incubated with whole skin digestion solution (RPMI medium, Liberase™ TL enzyme (Merck) at 0.1 mg/mL and DNAse 100x), for 2 hours at 37 ºC in a 6-well plate. Then, 0.25% Trypsin-1 mM EDTA (Gibco™) was added, in the last 10 minutes of the incubation step, followed by cold 5% FACS buffer to deactivate the enzymatic reaction. The skin suspension was further mechanically dissociated, passed through sterile 70 µm and 40 µm cell strainers, centrifuged at 400 *xg*, for 8 minutes at 4 ºC and the pellet was resuspended in PBS.

Furthermore, the skin immune population and glycosylation profile were characterized by FACS, using two different mixes of antibodies (**Table 2.2**). The first one is composed of FVD (1:2000), GNA (1:1000), SNA (1:1000), Streptavidin (1:200), EpCam (1:800), CD45 (1:500), B220 (1:400), TCRγδ (1:200), CD69 (1:200), CD25 (1:400), CD4 (1:400) and CD8 (1:500). This mix allows the identification of non-immune skin cells ($CD45^-$, $EpCam^+$), and the respective glycome characterization by lectins (GNA and SNA), and immune cells ($CD45^+$), which comprises $CD4^+$, $CD8^+$ and γδ T cells (with activation markers CD69 and CD25) and B cells ($B220^+$). The second mix allows the fibrocytes characterization, being composed by FVD (1:2000), TCRβ (1:300), B220 (1:100), CD45 (1:500), CD34 (1:100), αSMA (1:100), CD86 (1:100), MHC II (1:800), MRC2 (1:100) and Donkey α-rabbit IgG (1:100). The staining protocol is similar to the previous described however, in this case, the cells were stained for FVD alone for 30 minutes at 4 ºC, followed by fixation with 2% PFA and, on the following day, stained for lectins (15 minutes) and antibodies (30 minutes). Data acquisition was done Aurora cytometer (Cytec®) and files were analysed in FlowJo Software v.10.

Table 2.2 | Antibodies and lectins used in flow cytometry for VT1 aging mice skin characterization, their reactivity, clone, fluorochrome and supplier. Cy (cyanine), PE (phycoerythrin), APC (allophycocyanin).

Reactivity	Antibodies/ Lectins	Clone	Fluorochrome	Supplier
-	FVD	-	efluor 780	eBioscience
-	GNA	-	-	Vector Laboratories
-	SNA	-	Cy5	Vector Laboratories
Human, Mouse, Rat	Streptavidin	-	PE	BioLegend
Mouse	EpCAM	G8.8	Brilliant Violet 421	BioLegend
Mouse	CD45	30-F11	Alexa Fluor 700	BioLegend
Human, Mouse	B220	RA3-6B2	eFluor 506	eBioscience
Mouse	TCRγδ	eBioGL3	SuperBright 780	eBioscience
Mouse	CD69	H1.2F3	PE-Dazzle 594	BioLegend
Mouse	CD25	PC61.5	PE-Cy5.5	eBioscience
Mouse	CD4	RM4-5	Brilliant Violet 650	BD Biosciences
Mouse	CD8	53-6.7	PE-Cy7	eBioscience
Mouse	TCRβ	H57-597	Brilliant Violet 421	BioLegend
Mouse	B220	RA3-6B2	Brilliant Violet 421	BD Biosciences
Mouse	CD34	MEC14.7	APC	BioLegend
Human, Mouse, Rat	αSMA	1A4	Alexa Fluor 488	eBioscience
Mouse	CD86	GL-1	Brilliant Violet 510	BioLegend
Mouse	MHC II	M5/114.15.2	PE-Cy5	eBioscience
Human, Mouse	MRC2	-	-	Novus
Donkey	α-rabbit IgG	-	PE	Biolegend

2.10. Statistical analysis

All data was analysed with GraphPad Prism v7 software, and statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test, and Multiple T-test for multiple comparisons. The applied statistical test is mentioned in the respective figure's description. Statistical significance was considered with p-value < 0.05: *p ≤ 0.05, **p≤ 0.01, ***p ≤ 0.001).

3. RESULTS

3.1. HS and CD patients share a common abnormal glycosignature, characterized by an accumulation of high mannose *N*-glycans

To better understand the pathogenesis of HS and CD, we have characterized the glycosylation profile of skin and colon, respectively.

In collaboration with NYU Langone Health (New York, USA), we analyzed the RNAseq data (**Figure 3.1**) performed on tissue samples from HS patients. We have observed a down-regulation of *MGAT1* in non-immune cells (e.g., CD45 negative cells).

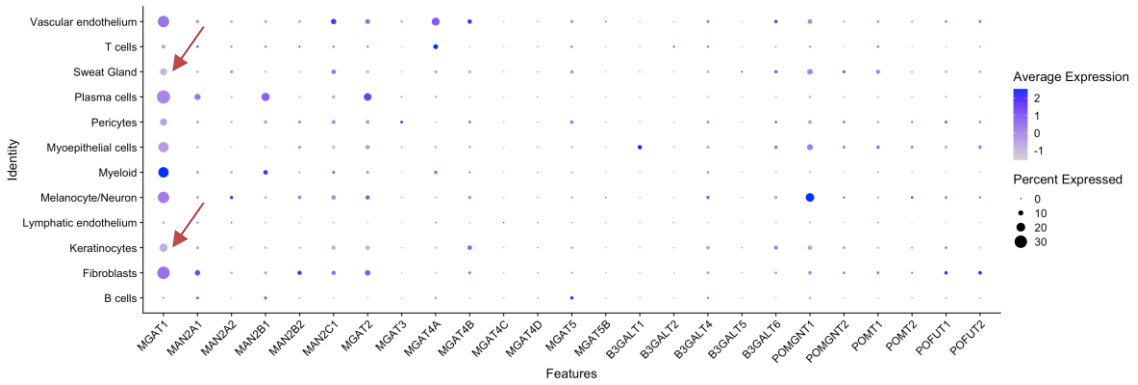

Figure 3.1 | RNA seq data of CD45 negative cells from HS patients, performed in collaboration with NYU Langone Health (New York, USA). Red arrows indicate the skin cells where the down-regulation of *MGAT1* is more pronounced (e.g., sweat glands and keratinocytes).

To validate whether this alteration in glycogenes expression is associated with changes in cellular glycosylation, we characterized the *in situ* glycan profile of HC, HS, and CD patients, using FFPE human biopsy samples from both skin and colon, respectively. We performed lectin histochemistry with GNA (a lectin that recognizes mannose-enriched glycans) and immunohistochemistry with Mannitou (a monoclonal antibody that recognizes *S. cerevisiae*-like glycans, usually mannan structures, and patients' isolated ASCA antibodies).

We have observed an overall increased reactivity of GNA in HS patients, compared with the HC, suggesting an accumulation of high mannose *N*-glycans in this tissue. Regarding Mannitou, we have also observed an increased reactivity in HS biopsies, possibly related to the accumulation of paucimannose glycans associated with the disease. Similar results were also observed in CD patients, however with a lower extent for both GNA and Mannitou reactivity (**Figure 3.2**).

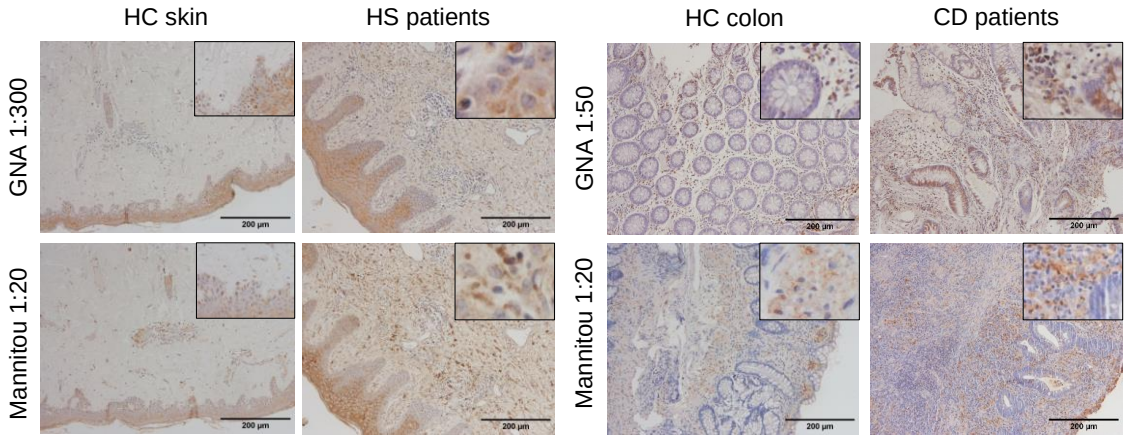

Figure 3.2 | Enrichment of high-mannose *N*-glycans and paucimannose glycans in HS patients. Representative images of skin and colon (ampliation 10x and 20x) upon histochemistry for GNA (recognizes high-mannose *N*-glycans) and Mannitou (recognizes ASCA epitopes). GNA and Mannitou both display increased expression in HS patients and, however to a lower extent, also in CD patients. GNA and Mannitou dilutions are described in Figure.

Interestingly, these results are in accordance with the RNAseq data (**Figure 3.1**), since a down-regulation of *MGAT1* in non-immune cells is accompanied by an increased reactivity of both GNA and Mannitou, suggesting an abnormal accumulation of oligomannosidic epitopes in the skin of HS patients, mostly in the epidermis. Moreover, the histopathological analysis of HS tissue samples revealed, as expected, an extensive infiltration of immune cells, that may be related to an abnormal and exacerbated immune response, together with a higher epidermal thickness and hyperkeratosis, typical of skin lesions in the context of HS.

Furthermore, the lectin histochemistry reactivity was evaluated by three independent observers (**Figure 3.3**) and in a quantitative analysis using ImageJ v8 (**Appendix 2**), in two different approaches: all tissue and by tissue components (epithelial and stromal compartments). The qualitative analysis revealed an overall higher reactivity of GNA and Mannitou in HS patients, more predominantly in the epithelial compartment, which is in accordance with the RNAseq data. Moreover, the quantitative analysis also showed an increased reactivity for both GNA and Mannitou in HS patients, although significant alterations were only detected when evaluating skin all tissue and stromal compartments. Unfortunately, in the CD cohort, these differences are not so striking, suggesting the need to increase the number of cases.

Although qualitative analysis is a subjective evaluation, it enables the correct identification of morphological skin sections (epithelial and stroma), allowing a more accurate quantification of these skin compartments. On the contrary, although quantitative analysis is a valuable tool, the proper selection of skin sections is not completely ensured, possibly resulting in data bias. Nevertheless, both histochemistry and quantification data are all in accordance, suggesting once more the accumulation of high mannose *N*-glycans and paucimannose glycans in these autoimmune diseases, although more significantly to HS.

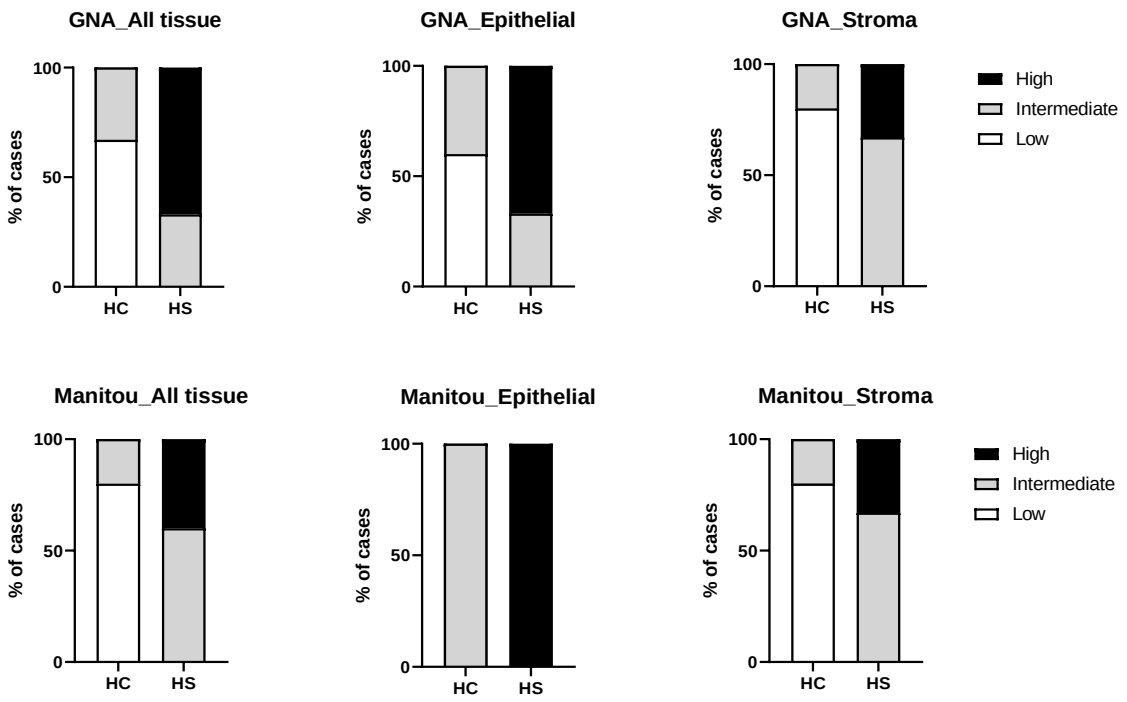

Figure 3.3 | Qualitative analysis of lectin reactivity of GNA and Mannitou histochemistry. Qualitative quantification for GNA and Mannitou histochemistry in HC and HS patients. The quantification was performed by three independent observers and different levels of reactivity were considered: Low (0-25%), Medium (25-75%), and High (> 75%).

To further validate this *in situ* characterization, we analysed the glycoprofile at the transcriptional level. For that, the expression of target genes related to glycosylation in HC, HS, and CD patients was assessed by RT-qPCR. The target glycogenes include the *MGAT1* (encodes for GnT-I that catalyzes the first addition of β1,2GlcNAc residue to the mannose arm), *MAN2A1* (encodes for alpha-mannosidase II, responsible for the removal of the terminal α1,3Man and α1,6Man residues, after GnT-I), *MGAT5* (encodes for GnT-V, responsible for β1,6GlcNAc branched *N*-glycans), *POMT1* (*O*-Manosylated glycans) and *GAPDH/HPRT* (Housekeeping gene).

Concerning the HS cohort, the results (**Figure 3.4.A**) showed a tendency for a down-regulation of *MGAT1* and a significant decrease for the *MAN2A1* gene, suggesting an accumulation of high mannose *N*-glycans due to the impairment of the glycosylation pathway in the first steps. On the other side, both *MGAT5* and *POMT1* show a tendency to be upregulated with no statistical significance. Regarding the CD cohort (**Figure 3.4.B**), the mRNA expression levels are similar between HC and CD patients in all the tested glycogenes, so no conclusions can be pointed out.

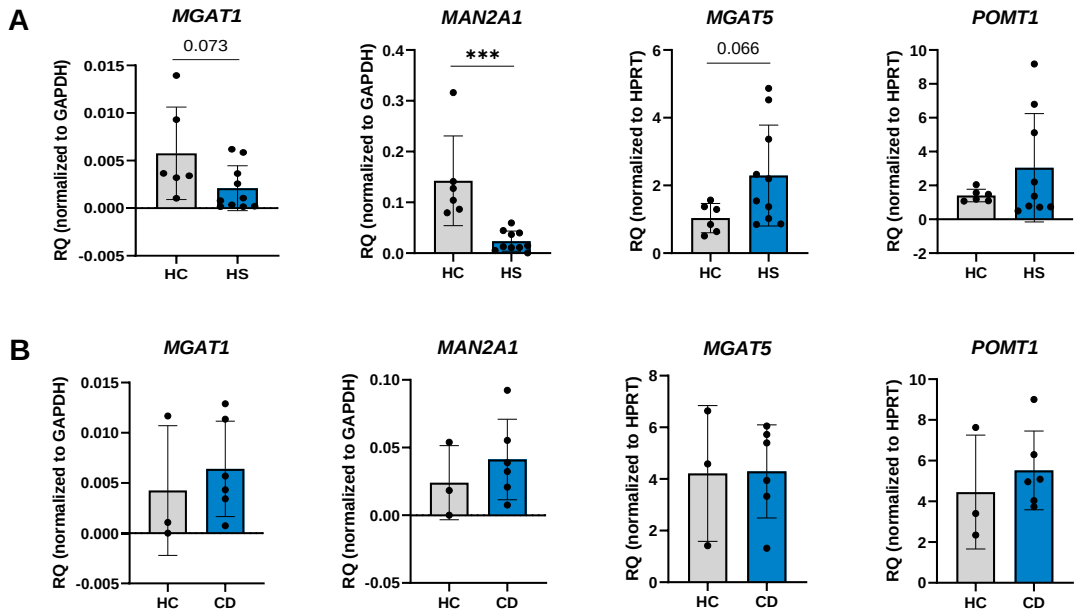

Figure 3.4 | *MAN2A1* mRNA expression levels are significantly lower in HS patients. (A) Relative expression of *MGAT1, MAN2A1, MGAT5 and POMT1* in HC (n=6) and HS (n=10). A down-regulation of the *MAN2A1* gene suggests an abnormal exposure of high mannose *N*-glycans in these patients. **(B)** Relative expression of *MGAT1, MAN2A1, MGAT5 and POMT1* in HC (n=3) and CD (n=6), with no statistical alterations in all the tested glycogenes. *MGAT1* and *MAN2A1* mRNA expression was normalized for the *GAPDH* housekeeping gene, and *MGAT5* and *POMT1* for the *HPRT* gene. Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: ***p ≤ 0.001.

Taking this into account, we can assume that the RT-qPCR results support the *in situ* glycan characterization of both skin and gut from HS and CD patients, respectively. Moreover, the abnormal exposure of oligomannosidic *N*-glycans, mainly in HS patients, may constitute a triggering event in the pathogenesis of this IMID that, ultimately, may stimulate the overproduction of anti-glycans antibodies, leading to pro-inflammatory responses in skin lesions of HS patients.

Moreover, as described by Möginger *et al.* [112], the healthy human skin *N*-glycome is enriched in biantennary fucosylated and sialylated *N*-glycan structures, in which the relative abundance of α2,3- and α2,6-linked NeuAc residues is similar. Taking this into consideration, we performed a histochemistry assay for SNA (a lectin that recognizes the α2,6-linked sialic acid) as a proof-of-concept (**Figure 3.5.A**). Among all tested HC and HS samples, only two cases of each cohort showed reactivity towards SNA and the subsequent lectin reactivity was shown to be higher in HC, mostly in the stromal compartment. Interestingly, these results are in accordance with the literature, although further histochemistry optimizations may be required. Furthermore, the SNA reactivity was quantitatively assessed, as previously performed, although no significant differences were detected (**Figure 3.5.B**). Nevertheless, in the two HC and HS cases that displayed reactivity towards SNA (identified as red dots in the quantification plot), the quantification analysis supports the histochemistry observations, in which a higher lectin reactivity is observed in HC when considering all tissue and stromal compartments.

Additionally, since the *N*-glycosylation is a dynamic pathway, we decided to evaluate how GNA and Mannitou levels are modified depending on SNA levels, through GNA/SNA and Mannitou/SNA ratios in both HC and HS context (**Figure 3.5.C**). Concerning the HC, both ratios are lower or equal (when considering all tissue quantification) to 1, meaning that the controls that do have more SNA, express lower levels of both GNA and Mannitou, as described in the literature. On the other hand, these ratios are significantly higher in HS patients, suggesting the accumulation of mannose-enriched *N*-glycans, and a subsequent lower content of sialylated structures in the context of disease, which supports our previous observations.

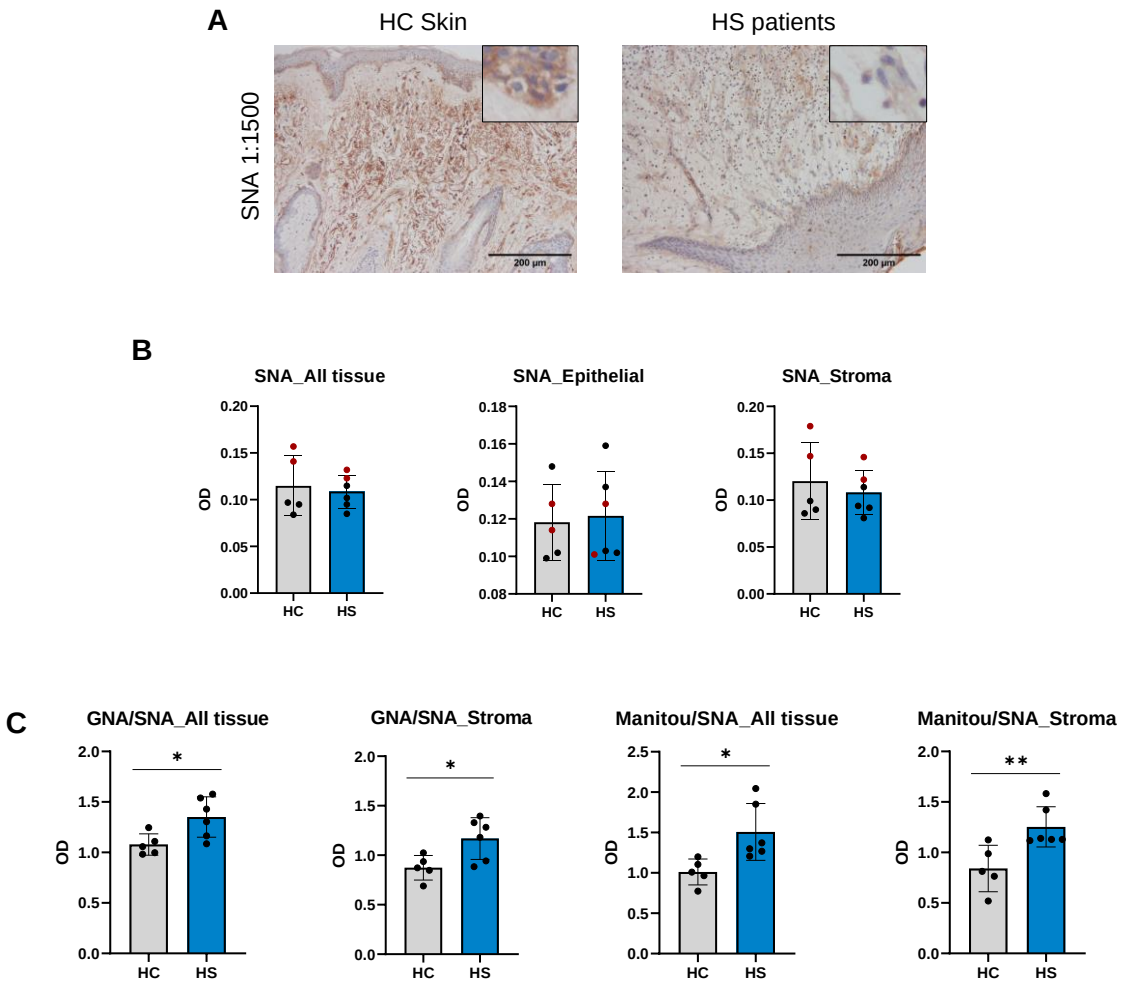

Figure 3.5 | SNA histochemistry as a proof-of-concept supports the accumulation of mannose-enriched *N*-glycans in HS. (A) Representative images of skin (ampliation 10x and 20x) upon lectin histochemistry for SNA (recognizes the α2,6-linked sialic acid). SNA dilution is described in Figure **(B)** Schematic representation of the quantitative evaluation of SNA histochemistry, calculated with ImageJ v8, from HC (n=5) and HS patients (n=6) in three subcategories: all tissue, epithelial and stromal compartments. The expression levels of SNA in HC and HS patients are similar, except for the two cases that showed reactivity towards SNA (identified as red dots). **(C)** GNA/SNA and Mannitou/SNA ratios for HC and HS. For the HC, both ratios are equal or lower than 1, in contrast with the HS patients. Thus, the HC that expresses higher levels of SNA, have lower levels of GNA and Mannitou, contrary to the HS patients. Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: *p ≤ 0.05, **p≤ 0.01.

Additionally, we analysed the expression of *ST6Gal1*, the gene that encodes for ST6 β-Galactoside α2,6-Sialyltransferase 1, in both HC and HS patients (**Figure 3.6**). Our data shows that HC tends to display increased *ST6Gal1* mRNA levels, possibly related to the accumulation of α2,6-linked NeuAc residues in healthy individuals, as described by Möginger *et al* [112], although not significantly. Importantly, this data recapitulates the SNA histochemistry results, reinforcing the accumulation of mannose-enriched *N*-glycans and the decrease of sialylated structures in the context of HS.

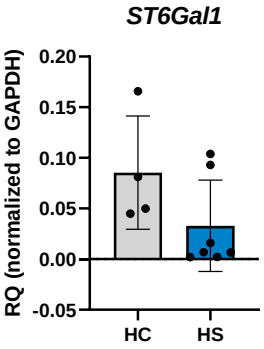

Figure 3.6 | HS patients display a tendency for a down-regulation of the *ST6Gal1* gene. Relative expression of *ST6Gal1* in HC (n=4) and HS (n=7). Statistical significance was assessed by the Mann-Whitney T-test: not significant.

3.2. Increased levels of anti-Man$_9$ antibodies are a common feature among HS and CD patients

Recent studies of our group have revealed a unique glycosignature of autoimmune-associated tissue lesions, such as kidney in lupus nephritis and skeletal muscle in IIM. This altered glycoprofile is characterized by an abnormal exposure of high-mannose *N*-glycan residues, which was associated with disease severity and susceptibility.[104,113] Concordantly, these glycans may constitute the epitopes to circulating antibodies that characterize several IMIDs, including HS and CD. In order to clarify the mechanism underlying the altered glycoantigens expression (high mannose *N*-glycans and paucimannose glycans) associated with inflammatory conditions and the loss of immunotolerance, we have characterized the glycoantigens' recognition by antigen-specific IgGs from HS and CD patients.

For that, we performed an ELISA assay to understand the affinity and specificity of total IgGs from HS and CD patients towards different glycoantigens: OVA-Man$_9$ and OVA-diGlcNAc. Therefore, Man$_9$ and diGlcNAc structures, which respectively mimic high mannose *N*-glycans and branched *N*-glycans, were previously conjugated to Ovalbumin (OVA). Concerning the HS patients (**Figure 3.7.A**), and in a concentration-dependent manner, the recognition of both OVA-Man$_9$ and OVA-diGlcNAc is indeed specific, as the resulting plot is an affinity curve. However, this interaction is stronger for OVA-Man$_9$, suggesting that IgGs from HS patients have a higher affinity towards mannosylated antigens. Regarding CD patients (**Figure 3.7.B**), although the interaction between IgGs and glycoantigens is not so specific compared to the HS cohort (less hyperbolic behaviour), similar results can also be pointed out.

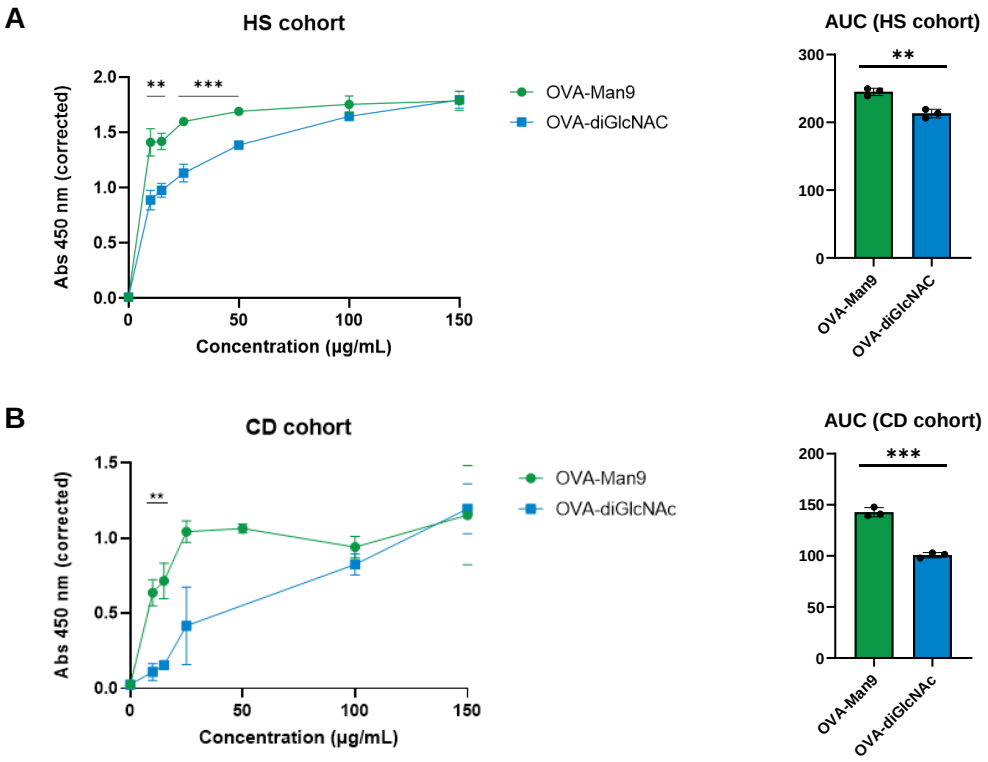

Figure 3.7 | Total IgGs from HS and CD patients have a higher affinity towards mannosylated antigens. (A) ELISA assay with total IgGs from HS patients towards OVA-Man_9 and OVA-diGlcNAc, and the corresponding area under the curve (AUC) plot. We did a pool of total IgGs from 8 patients and divided it by 3 (n=3), so each dot represents the average of 3 technical replicates. **(B)** ELISA assay with total IgGs from CD patients towards OVA-Man_9 and OVA-diGlcNAc, and the corresponding AUC plot. We did a pool of total IgGs from 5 patients and divided it by 3 (n=3), so each dot represents the average of 3 technical replicates. In both assays, increasing concentrations of glycoantigens were used: 0, 10, 15, 25, 50, 100, and 150 µg/mL. Statistical significance was assessed by Multiple T-tests for multiple comparisons: **p≤ 0.01, ***p≤ 0.001.

Afterwards, we decided to check the levels of anti-Man_9 and anti-diGlcNAc antibodies in HS and CD patients, through an ELISA assay for OVA-Man_9 and OVA-diGlcNAc at a concentration of 10 µg/mL. Additionally, we decided to use samples from HC, CD, HS, SLE, IMM, Sj, and isolated IgGs from HC and SLE Man_9-specific B cells, to check if this could be a common feature across several autoimmune pathologies (**Figure 3.8**). Interestingly, in comparison to the HC, IMIDs patients tend to display increased levels of anti-Man_9 antibodies, possibly due to a common abnormal glycoprofile mostly composed of mannose-enriched *N*-glycans. This preliminary evidence pinpoints common pathological events regarding autoimmunity, essentially characterized by the exposure of immunogenic high-mannose *N*-glycans residues. Importantly, anti-GlcNAc antibodies should not be considered as a control to characterize and compare the prevalence of anti-Man_9 antibodies in IMIDs, since anti-GlcNAc antibodies are described to be naturally present in mammals.[114] In fact, natural antibodies are produced without external antigen stimuli, which are important to assist the immune system in avoiding the infection.[115] With this in mind, it is important to consider an alternative control to exclude the effect of OVA, as well as to increase the number of cases in this assay.

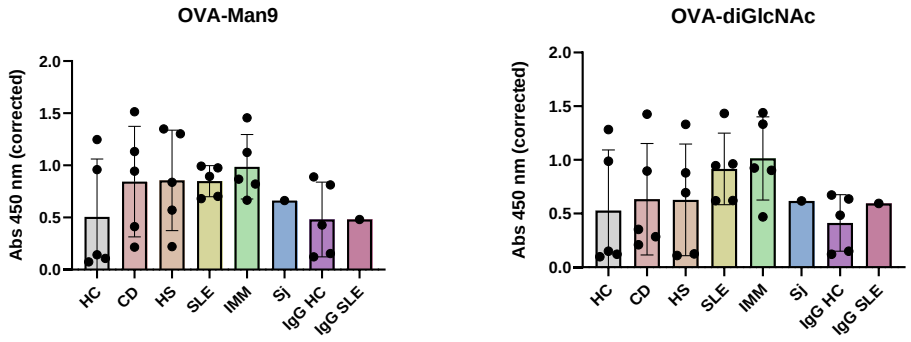

Figure 3.8 | Increased levels of anti-Man_9 antibodies are a common feature across several IMIDs. Schematic representation of ELISA assay towards OVA-Man_9 and OVA-diGlcNAc (10 µg/mL) of total IgGs from different IMIDs: HC (n=5), CD (n=5), HS (n=5), SLE (n=5), IMM (n=5), Sj (n=1), and isolated IgGs from HC (n=5) and SLE (n=1) Man_9-specific B cells. Statistical significance was assessed by the Mann-Whitney T-test: not significant.

Altogether, these results indicate that HS and CD patients present antibodies with a greater affinity to mannosylated epitopes as well as a tendency for increased levels of anti-Man_9 IgGs in their serum, together with other IMIDs. Nevertheless, and although promising, this preliminary data requires further validation as we need to increase the sample size to better establish the different levels of anti-Man_9 IgGs in IMIDs, in comparison with HC.

3.2.1. Anti-Man$_9$ antibodies as potential drivers of Gut-Skin Axis

Given that both HS and CD patients display high titters of serological anti-Man$_9$ antibodies, we aimed to determine if these could establish a novel gut-skin axis and an immunological link between both autoimmune disorders.

With this in mind, we did a western blot assay with colonic proteins from HC and CD with IgGs from either HC (**Figure 3.9.A**) or HS patients (**Figure 3.9.B**), followed by a quantitative analysis in Image Lab software (**Figure 3.9.C**). This assay would allow us to infer the cross-reactivity of anti-glycan antibodies from HS patients to colonic CD tissue. Importantly, the quantification graph results from the normalization of HS IgGs to HC IgGs blot, thus making it possible to see the effect of IgGs, without considering the self-tissue reactivity. These preliminary results indicate that antibodies from HS patients tend to have a higher cross-reactivity to proteins from CD patients, although not significantly. Afterwards, this data indicates that IgGs from HS can also recognize colonic proteins from CD patients, suggesting that skin and colon may share common epitopes, important for the establishment of a gut-skin axis.

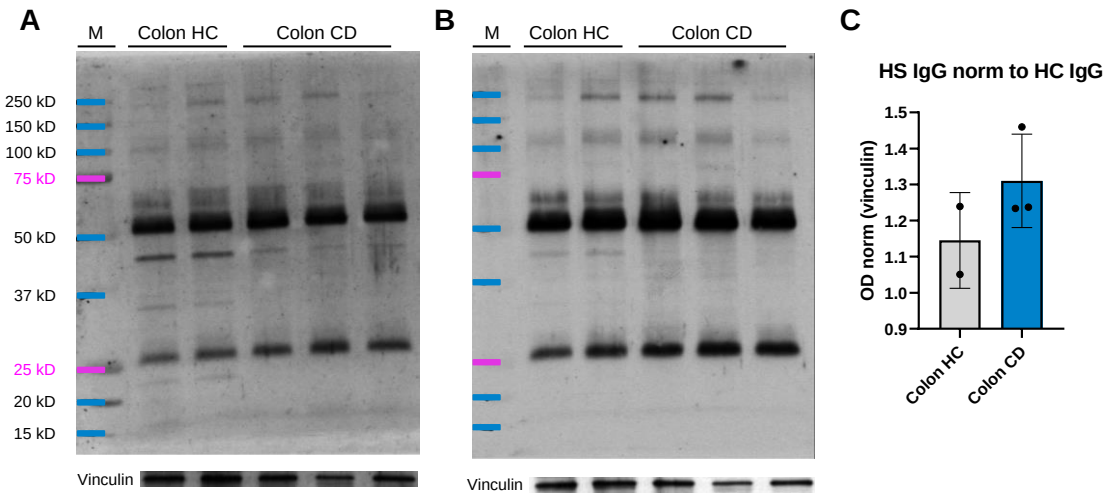

Figure 3.9 | Antibodies from HS patients tend to have a higher cross-reactivity with colonic proteins from CD patients. (A) Western blot assay with colonic proteins from HC and CD patients with IgGs from HC, the respective vinculin as the loading control and the Dual Color as protein marker (Bio-Rad Laboratories). **(B)** Western blot assay with colonic proteins from HC and CD patients with IgGs from HS, the respective vinculin as the loading control and the Dual Color as protein marker (Bio-Rad Laboratories). **(C)** Schematic representation of quantitative analysis from the HS IgGs blot normalized to the HC IgGs (individual plots are depicted in Appendix 3). This quantification analysis was performed with Image Lab software. Statistical significance was assessed by the Mann-Whitney T-test: not significant.

3.3. The potential role of fibrocytes, a novel immune cell population, in the immunopathogenesis of HS

RNAseq data from HS tissue samples, performed in collaboration with NYU Langone Health (New York, USA), highlighted an overexpression of MRC2 (the gene that codes for Mannose Receptor type II, CD280) in a subtype of $CD45^+$ fibroblasts, which are commonly known as fibrocytes (**Figure 3.10**). This observation led us to characterize the impact of this novel immune cell population in its capacity to recognize glycoantigens associated with the immunopathogenesis of HS.

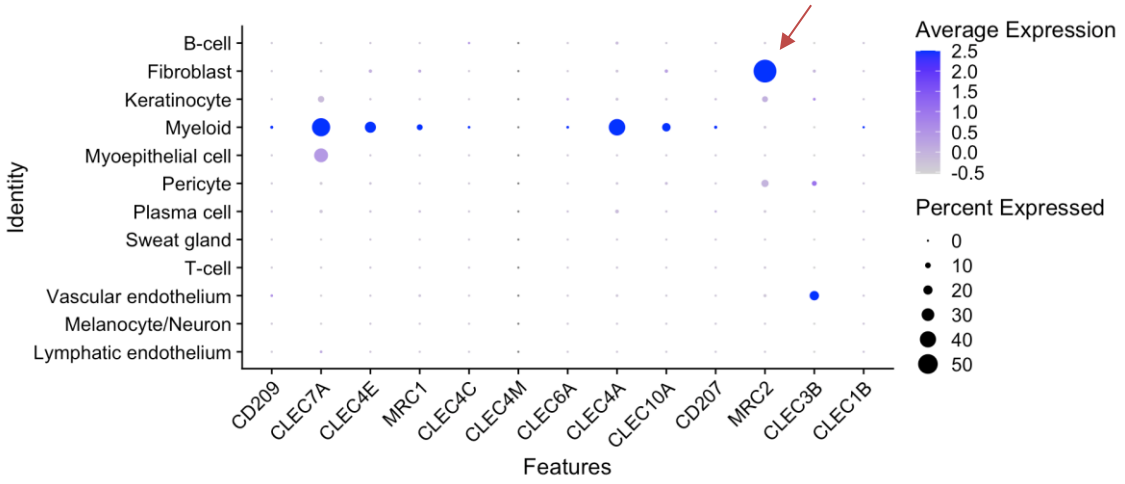

Figure 3.10 | RNA seq data of CD45 positive cells from HS patients. The red arrow indicates a population of $CD45^+$ fibroblasts that express high levels of MRC2 (mannose receptor type 2).

We have characterized the expression of MRC2 in fibrocytes, taking into consideration the presence of its ligand, high-mannose *N*-glycans, and to check how this interaction would modulate the expression of MRC2 and the fibrocytes activation, depending on the glycoantigen exposed. Thus, fibrocytes were collected after 10 days of continuous culture and then cultured with OVA-Man_9 and OVA-diGlcNAc for 3 days, as described in the Materials & Methods section.

Fibrocytes were identified by FACS as $CD45^+$, $CD34^+$ and αSMA^+ cells, excluding the lineage-negative (LN) composed of IgM^+, $CD3^+$ and $CD14^+$, corresponding to B cells, T cells and monocytes, respectively (**Figure 3.11.A**). We have observed a significantly increased expression of MRC2 (MFI MRC2) in fibrocytes incubated with OVA-Man_9, as well as a higher frequency of $MRC2^+$ fibrocytes, as expected. Besides MRC2, we also observed a significantly higher frequency of MHC II^+ fibrocytes and a tendency for higher levels of CD86, an important co-stimulatory molecule (**Figure 3.11.B**). Both MHC II and CD86 are general characteristics of APCs, demonstrating that fibrocytes display increased levels of antigen presentation upon interaction with OVA-Man_9 via MRC2.

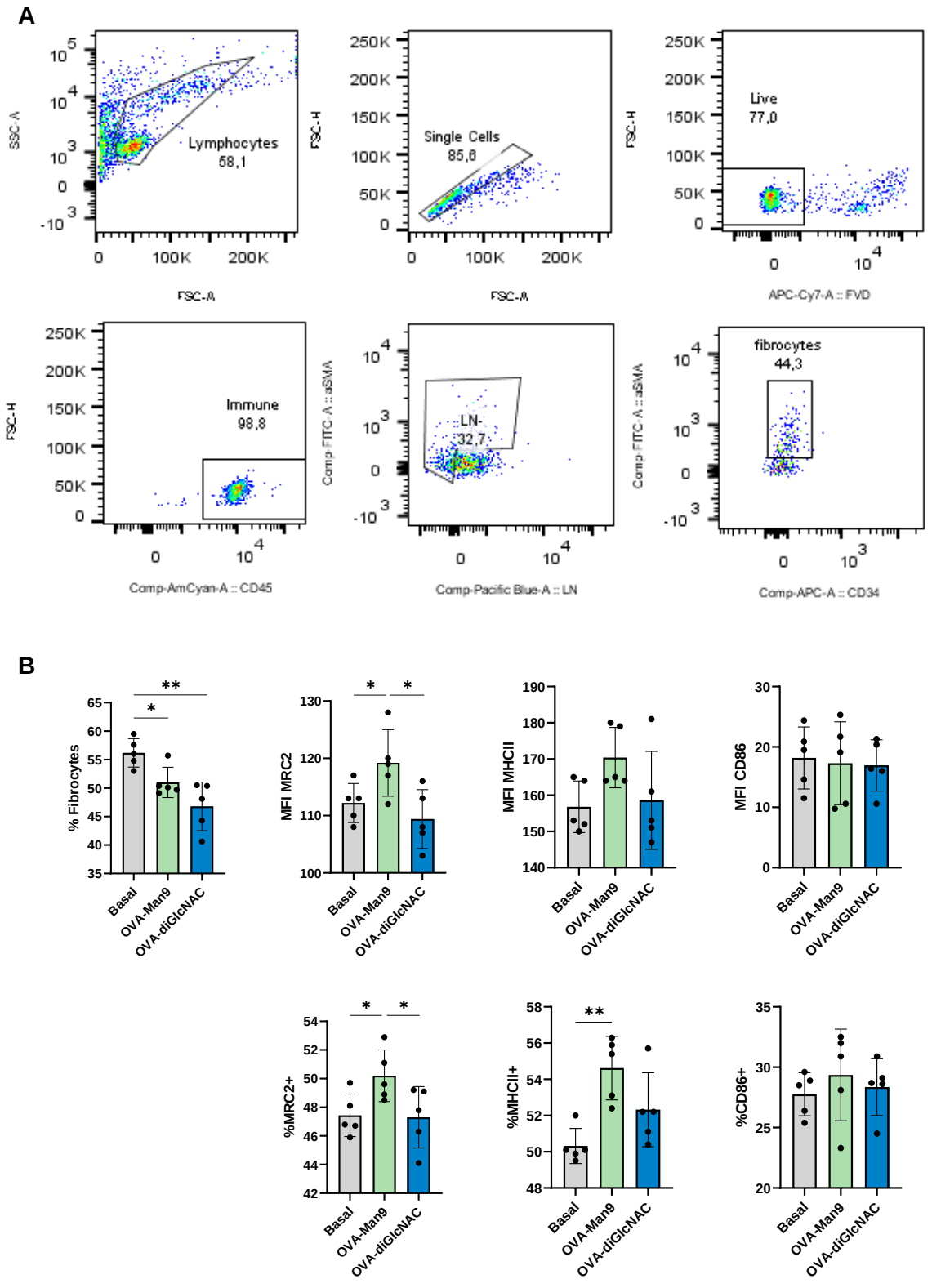

Figure 3.11 | Fibrocytes express higher levels of MRC2 and MHC II upon interaction with OVA-Man$_9$. **(A)** Gating strategy in FACS analysis of fibrocytes (IgM$^-$, CD3$^-$, CD14$^-$, CD45$^+$, CD34$^+$ and αSMA$^+$). **(B)** Schematic representation of total %fibrocytes, MFI (median fluorescence intensity) of MRC2, MHCII and CD86 of fibrocytes population, and frequency of MRC2$^+$, MHCII$^+$ and CD86$^+$ fibrocytes, upon interaction with OVA-Man$_9$ and OVA-diGlcNAc. Note that, each dot represents a technical replicate of the experiment (n=5). Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: *p ≤ 0.05, **p≤ 0.01.

Additionally, we decided to analyse the cytokine profile of the fibrocytes incubated with glycoantigens, to determine whether the interaction with mannosylated structures would induce a more pro-inflammatory phenotype of fibrocytes. With this in mind, we tested several cytokines (IL-1β, TNF, IL-8, IL-6, IL-10 and IL-12p70) but, unfortunately, the results were not very enlightening, since the concentration of cytokines in the three groups (Basal, Ova-Man_9 and OVA-diGlcNAc) is very similar (**Figure 3.12**). The only exception is for IL-1β, although the concentration values of OVA-diGlcNAc supernatants were lower than the interval range set by the calibration curve. Nevertheless, these results indicate that fibrocytes when in contact with mannosylated epitopes tend to secrete IL-1β, which is in fact an important pro-inflammatory cytokine in the pathogenesis of HS. Although preliminary, this assay must be repeated with isolated fibrocytes (e.g., sorted $CD45^+$, $CD34^+$ and αSMA^+ cells), as the presence of other immune cell subsets in the culture may bias the data.

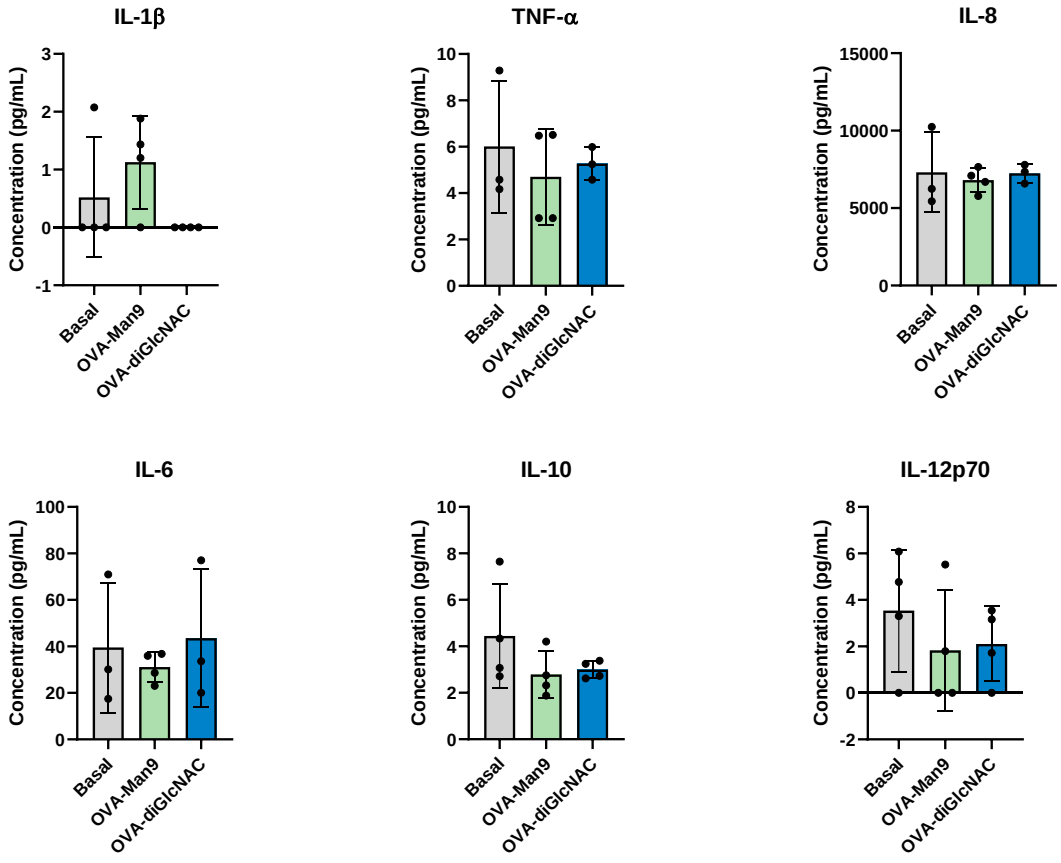

Figure 3.12 | Fibrocytes in culture with OVA-Man_9 do not express higher levels of pro-inflammatory cytokines. This analysis was performed with a commercial kit from BD Biosciences (BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokine) and six inflammatory cytokines were analysed: IL-1β, TNF, IL-8, IL-6, IL-10 and IL-12p70. Each dot represents a technical replicate (n=4). Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: not significant.

3.4. *VT1*$^{-/-}$ aging mice display high titers of serological ASCA-IgGs and lower levels of circulating fibrocytes in the skin

To better characterize the gut-skin axis that we are here hypothesising, we tested whether the exposure of abnormal high-mannose *N*-glycans in the gut would promote alterations in the skin (e.g., glycome, skin lesions, inflammation), either mediated by fibrocytes and/or ASCA-IgGs, following the monitorization of glycoengineered mice model with susceptibility to colitis. This mice model (VT1) is a Cre conditional KO of the *Mgat1* gene in villin-positive cells from the small intestine and colon, where the abolishment of *Mgat1* expression results in the accumulation of high mannose *N*-glycans at the surface of gut epithelia. To amplify the phenotype and allow immune responses to be established, we let the mice age for 60-68 weeks.

With this in mind, we decided to quantify the levels of ASCA in the serum of WT (*VT1*$^{+/+}$) and KO (*VT1*$^{-/-}$) VT1 aging mice, through an ELISA assay. Interestingly, significantly higher levels of ASCA-IgGs were detected in the KO mice (**Figure 3.13**), suggesting that the abnormal exposure of mannosylated antigens in the gut promotes the synthesis of auto-reactive anti-glycan antibodies.

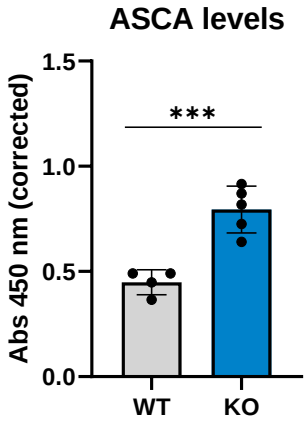

Figure 3.13 | *VT1*$^{-/-}$ aging mice display high titers of serological ASCA-IgGs. The quantification of ASCA levels was performed in an ELISA assay (QUANTA Lite® ASCA (*S.cerevisiae*) IgG Kit, Werfen), including both *VT1*$^{+/+}$ (n=4) and *VT1*$^{-/-}$ (n=5) aging mice with 60-68 weeks. Statistical significance was assessed by the Shapiro-Wilk test: ***p ≤ 0.001.

Afterwards, the skin from both *VT1*$^{+/+}$ and *VT1*$^{-/-}$ aging mice was characterized by histopathological and FACS analysis, including skin immune population, skin glycome and skin morphology. Regarding the histological analysis (**Figure 3.14**), mice's skin displays a healthy-like phenotype, without morphological lesions typically found in HS patients. However, some subtle evidences of inflammation on the *VT1*$^{-/-}$ skin can be observed such as slightly increased epidermal thickness and slightly higher presence of immune cells in the KO mice.

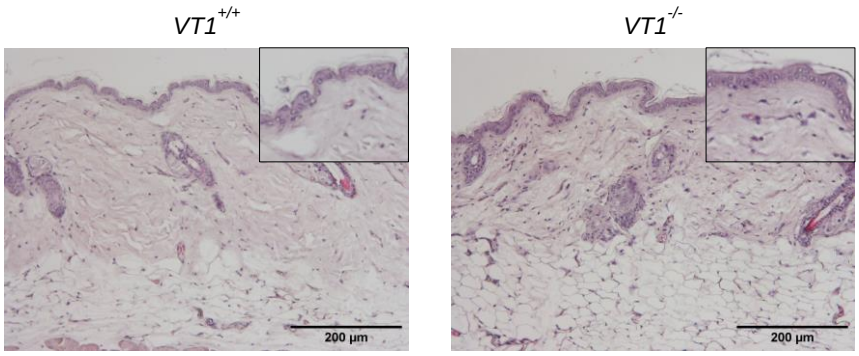

Figure 3.14 | The skin from *VT1*$^{-/-}$ aging mice does not show signs of lesions and inflammation, in comparison with *VT1*$^{+/+}$ mice. Representative images of skin from *VT1*$^{+/+}$ and *VT1*$^{-/-}$ aging mice, after HE staining (Ampliation: 10x, 20x).

Moreover, a skin single-cell suspension from *VT1*$^{+/+}$ and *VT1*$^{-/-}$ aging mice was obtained, followed by FACS characterization of both immune (CD45^{+}) and non-immune (CD45^{-}) components. Concerning the non-immune cells (**Figure 3.15**), namely epidermal (epithelial cell adhesion molecule, EpCAM) cells, we decided to characterize the glycosylation profile by GNA and SNA lectins and no significant alterations were detected between WT and KO mice.

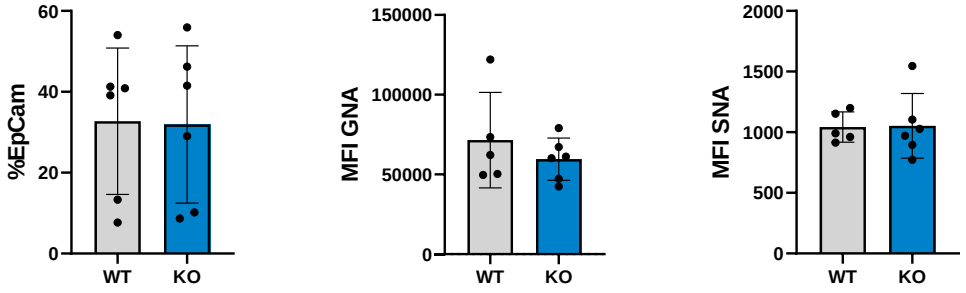

Figure 3.15 | The skin glycome in both WT and KO VT1 aging mice is similar. This analysis was performed by FACS and includes the glycan profile characterization of skin CD45^{-} cells by GNA (PE-streptavidin) and SNA lectins that recognize α1,3Man and α2,6-linked sialic acid, respectively. This analysis includes both *VT1*$^{+/+}$ (n=6) and *VT1*$^{-/-}$ (n=6) aging mice. Statistical significance was assessed by the Shapiro-Wilk test: not significant.

Regarding the skin immune population (**Figure 3.16**), KO mice tend to present slightly increased levels of CD45^{+} (immune cells), accordingly with our histological observations, including a tendency for increased levels of CD4^{+} T cells and B cells, contrary to CD8^{+} and γδ T cells (schematic representation of activation markers CD69 and CD25 in **Appendix 4**), although not significantly. Interestingly, the only significant alteration relies on the percentage of fibrocytes, which is markedly reduced in the skin of KO mice, which suggests a fibrocyte-dependent mechanism in this mice model, which requires further exploitation.

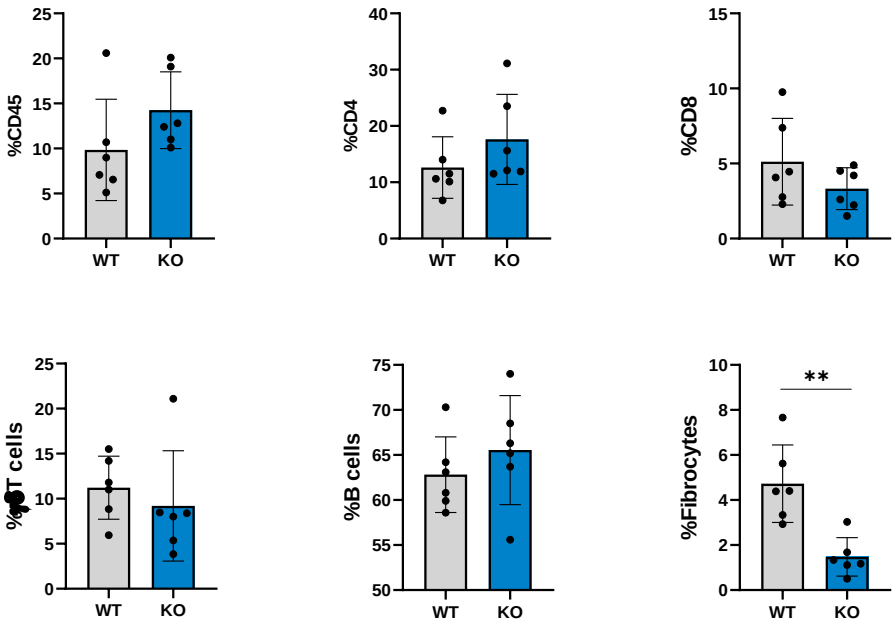

Figure 3.16 | The skin immune compartment (CD45^{+}) of *VT1*$^{-/-}$ aging mice is significantly less abundant in fibrocytes. This characterization, which comprises CD4^{+}, CD8^{+}, γδ T cells (identified as TCRγδ^{+} with activation markers CD69 and CD25, in Appendix 4), B cells (B220^{+} cells) and fibrocytes (TCRβ^{-}, B220^{-}, CD34^{+}, αSMA^{+}), was performed by FACS and includes both *VT1*$^{+/+}$ (n=6) and *VT1*$^{-/-}$ (n=6) aging mice. Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: **p≤ 0.01.

4. DISCUSSION

HS is a chronic inflammatory skin disorder, estimated to affect 0.05 - 4.10% of the worldwide population, with a higher incidence in young adults. HS is usually manifested by subcutaneous painful nodules in apocrine glands enriched areas, pus secretion, abscess and fistula formation.[7,13] Interestingly, HS is one of the extraintestinal manifestations of CD, which is characterized by a transmural inflammation of the gastrointestinal tract.[25,36] Nevertheless, the aetiology of HS remains largely unknown and its association with CD requires further exploitation, although some studies pinpoint a shared accumulation of ASCA antibodies in the serum of these patients.[32,38] Moreover, glycans are complex and diverse structures that decorate the surface of all cell types, being responsible for mediating a variety of cellular processes in both homeostatic and pathological conditions.[83,85] In fact, glycans contain an immense amount of biological information that is usually decoded by the immune system through their recognition by glycan-binding proteins.[94,99] However, in an autoimmune scenario, the exposure of altered host glycoantigens, usually composed of oligomannosidic structures, results in the loss of immune tolerance by a mechanism called glycan mimicry. With this in mind, we aim to unravel a potential common glycan-based mechanism underlying the immunopathogenesis of HS and CD. For that, we aimed to evaluate the effects of high levels of serological ASCA-IgGs in triggering pro-inflammatory response potentially mediated by fibrocytes, as a novel immune cell type implicated in chronic inflammation and autoimmunity.[71,73,76] Altogether we aim to evaluate the existence of a potential novel Gut-Skin axis responsible for the association of these IMIDs.

Our *in situ* results demonstrate increased levels of GNA and Mannitou reactivity, suggesting an abnormal accumulation of high-mannose *N*-glycans and paucimannose glycans in both HS and CD patients. These results are in accordance with the RNAseq data from HS patients, where a down-regulation of *MGAT1* is accompanied by an accumulation of oligomannosidic epitopes in the skin due to the breakdown of the *N*-glycosylation pathway.[89] Altogether, these preliminary results pinpoint a glycan switch event in the transition from health to an inflammatory/autoimmune phenotype, essentially characterized by an abundance of unusual high mannose *N*-glycans that are typically found in pathogens, therefore being recognized as PAMPs by the immune system.

In fact, pathogens display an immunogenic layer of mannose-enriched glycans on their surface, capable of inducing pro-inflammatory immune responses through glycan recognition by CTLs, namely DC-SIGN and MR. In the case of Fungi, namely *S. cerevisiae* and *Candida albicans*, the cell wall is usually composed of high-mannose *N*-glycans including mannan (β1,4Man), which is recognized by ASCA-IgGs, and β-glucan (β1,4 and β1,3 Glu) structures. Veerdonk *et al.*, has demonstrated that, during *C. albicans* infection, mannan structures can be recognized by MR, leading to a Th17 pro-inflammatory response, characterized by IL-17 secretion.[116] In addition, Dectin-2 is a GBP expressed by macrophages and DCs that recognizes α-mannan structures, important to establish an immune response against fungi, essentially

characterized by a Th17 pro-inflammatory response.[117] Afterwards, the mannose recognition by CTLs is also described to activate pro-inflammatory signalling pathways, including the secretion of IL-12 and IL-23 by DCs that will promote a Th1 (IFNγ) and Th17 (IL-17) immune response, respectively. This mechanism was described for a variety of autoimmune disorders including HS [61], RA [118], in which the abolishment of the IL-23/Th17 axis conferred protection against the development of CIA (collagen-induced arthritis) mice model, and SLE, where IL-17 was associated with a more severe and therapy-resistant phenotype of the disease.[119] In addition, Alves *et al.* have demonstrated that abnormal exposure of mannose-enriched glycans in the kidney of SLE patients enables its recognition by γδ T cells via DC-SIGN binding motifs, resulting in a pro-inflammatory response and kidney damage, mediated by IL-17.[104,105] Concordantly, mannosidase II deficient mice have developed lupus disease with kidney inflammation, dysfunction and increased levels of autoantibodies, revealing the importance of an altered glycosylation profile in the pathogenesis of this disease.[120]

All this evidence points to the high immunogenic potential of high-mannose *N*-glycans in mediating an immune response against pathogens and, in an autoimmunity scenario, against the host, where the accumulation of these glycans has been widely described in several IMIDs. This altered glycosignature makes it impossible to discriminate "self" from "non-self" antigens, leading to the loss of immune tolerance by a process called "glycan mimicry".[102] This may suggest that the abnormal exposure of highly mannosylated glycans can originate or perpetuate ASCA-mediated response, in which the epitopes are in fact high contents of mannose (mannan, as depicted in **Figure 2.1.C**).

Following the *in situ* glycoprofile characterization, we have analysed the mRNA levels of several glycogenes. Regarding the HS patients, a tendency for downregulation of *MGAT1* and a significant decrease of *MAN2A1* were observed, corroborating our *in situ* data. The lower mRNA levels of these glycogenes may be associated with a breakdown of the *N*-glycosylation pathway, culminating in the accumulation of mannose-enriched *N*-glycans, as already mentioned. Moreover, we also observed a tendency for higher *MGAT5* mRNA expression levels in HS, not necessarily related to the accumulation of branched *N*-glycans. In fact, this may illustrate a compensatory mechanism in the glycosylation pathway since GnT-I is not using its substrate, leading to increased availability of GlcNAc and, consequently, favouring the enzymes further down this pathway. Similar results can also be described in the CD cohort, although no significant alterations were detected, revealing the importance of increasing the number of patients in this cohort.

Additionally, Möginger *et al.* [112] have demonstrated that the healthy human skin *N*-glycome is enriched in biantennary fucosylated and sialylated *N*-glycans structures, comprising similar levels of α2,3- and α2,6-linked NeuAc residues. As a proof of concept, we decided to characterize the SNA reactivity and *ST6Gal1* mRNA levels in both HC and HS patients and, interestingly, our results are consistent with those published in the literature. Histochemistry analysis revealed an

increased reactivity of SNA in HC, compared to the HS patients, although this was only observed in two of all tested samples, highlighting the need to optimize the histochemistry protocol. The SNA reactivity was quantitatively assessed, and the results are in line with the histochemistry analysis. In fact, the two HC were found to display increased levels of reactivity, in comparison to HS, mostly in stromal compartments. In addition, the GNA/SNA and Mannitou/SNA ratios were calculated to be equal or lower than one in the case of HC, suggesting that the HC that display higher levels of SNA reactivity, express lower levels of both GNA and Mannitou. For the HS patients, both ratios were calculated to be higher than one, suggesting a lower abundance of sialylated structures and reinforcing the accumulation of mannose-enriched *N*-glycans in this disease. Regarding the transcriptional characterization, our results also support this evidence since a tendency for a down-regulation of *ST6Gal1* was detected in HS patients. Importantly, *Phaseolus vulgaris Leucoagglutinin* (L-PHA) lectin, which usually recognizes β1,6-GlcNAc branched *N*-glycans would not be suitable for this proof-of-concept since the healthy human skin is enriched in biantennary structures, and L-PHA recognizes tetraantennary glycans.[107]

Interestingly, the same authors also describe that the relative abundance of both high-mannose and paucimannosidic *N*-glycans in healthy human skin is almost negligible (5.19% and 1.98%, respectively).[112] This reinforces our hypothesis of a glycan switch event in the transition from health to an autoimmune disorder, although the exact mechanism underlying this altered host glycosylation remains to be elucidated. Nevertheless, some authors suggest that infection and pro-inflammatory cytokines (e.g., IFNγ) may constitute the trigger for this glycan switch, mainly through the impairment of the *N*-glycosylation pathway with the subsequent down-regulation of *MGAT1*, *MAN2A1* and *ST6Gal1*, as also demonstrated in our results.[121,122]

Although promising, these results are still preliminary due to the low number of cases of HS and CD comprising our cohort. Our effort will be to increase the number of cases to a larger validation cohort and to correlate the abnormal glycosignature with clinical variables, as already performed in previous studies.[104,113] In fact, it is essential to correlate the abnormal accumulation of high-mannose *N*-glycans with disease stage, activity and response to treatment (among others) to better understand the importance of these glycans in the context of disease.

Taking into consideration the increased expression of mannosylated epitopes at the tissue level, we aimed to demonstrate the respective levels of anti-glycan autoantibodies and their presence in CD and HS. To do so, we performed an ELISA assay to understand the affinity of total IgGs from HS and CD patients towards several glycoantigens, including OVA-Man_9 and OVA-diGlcNAc that simulate high-mannose and branched *N*-glycans, respectively. Our results indicate that IgGs from these patients have a higher affinity and specificity towards mannosylated structures, with concomitantly higher levels of serological anti-Man_9 antibodies. The origin and existence of this anti-glycan antibody have been already explored in the context of infection. Namely, Calarese *et al.* [123] have demonstrated that 2G12 broadly neutralising antibodies can recognize the α1,2Man motifs within the $Man_9GlcNAc_2$ structures present on the gp120 viral HIV

protein. Interestingly, 2G12 has been found to display cross-reactivity with mannan structures from *C. albicans* and *S. cerevisiae*, which is abundantly composed of α1,2Man with terminal α1,3Man residues.[124] Thus, the presence of α1,2Man residues in mannan resembles the glycoepitope recognized by 2G12, suggesting similar recognition domains and a glycan mimicry between both mannan and Man_9 structures.[125] Furthermore, Cambi *et al.* [126] have demonstrated that DCs, via DC-SIGN and MR, mediate the recognition and internalization of *N*-linked mannan residues of *C. albicans*, leading to the activation of an immune response to avoid the infection, mainly characterized by IL-6 production. Thus, the recognition of mannan residues by human DCs, through DC-SIGN and MR, reinforces the glycan mimicry between mannan and Man_9 residues, since these CTLs are described to recognize high-mannose *N*-glycans.[121]

Considering this evidence, we have hypothesized that the increased levels of anti-Man_9 antibodies in HS and CD patients can be compared to ASCA antibodies since Man_9 and mannan have similar recognition domains. In fact, it has already been described that both HS and CD patients display high titters of serological ASCA IgGs, a class of anti-microbial antibodies that usually recognizes oligomannosidic mannan epitopes in *C. albicans* and *S. cerevisiae*.[32,38] Therefore, we can assume that the exposure of abnormal high-mannose *N*-glycans in the skin/gut can be recognized by ASCA IgG because of glycan mimicry, possibly stimulating the overproduction of pro-inflammatory ASCA-IgG by B cells. Moreover, our group has already described that the abnormal exposure of mannosylated glycans is a common feature across several IMIDs.[104,105,113,127] Interestingly, the prevalence of ASCA has been described, not only in HS and CD, but in a variety of autoimmune disorders including SpA [128], Bençet's disease [129], and SLE.[130] This pinpoints a shared pathogenic mechanism across several IMIDs, characterized by an accumulation of high-mannose *N*-glycans and their subsequent recognition by ASCA antibodies, leading to the loss of immune tolerance by glycan mimicry. In addition to HS and CD patients, we also evaluated the levels of anti-Man_9 antibodies in other IMIDs including SLE, Sj and IIM. According to the literature, our results revealed a tendency for increased levels of anti-Man_9 antibodies that can be linked to the unusual accumulation of mannose-enriched *N*-glycans in autoimmunity, as already described. Nevertheless, this assay must be repeated with an increased number of cases and with a more suitable control, rather than the natural OVA-diGlcNAc antibodies, to exclude the effect of OVA.[114,115]

Since increased levels of anti-Man_9 antibodies were detected in both HS and CD, we decided to check if these antibodies could be involved in the association of both diseases and in a possible gut-skin axis. With this in mind, we performed a western blot with colonic TCL from HC and CD and antibodies from HC and HS patients. Our preliminary results suggest that IgGs from HS (highly reactive to mannosylated-enriched structures) can also recognize colonic protein from CD patients, suggesting that skin and colon may share common glycoepitopes. Thus, we can hypothesize that high titters of serological ASCA antibodies in both HS and CD context may underly a common biological pathway associated with HS and CD onset, therefore contributing to common pathogenic mechanisms and to a novel gut-skin axis, important for the

immunopathogenesis of these disorders. Nevertheless, it would also be interesting to test TCL from the skin of HC and HS patients and IgGs from HC and CD, to better establish the gut-skin axis that we propose. It is important to mention that these are preliminary results, that require further validation.

Afterwards, in the RNAseq data performed on tissue samples from HS patients, we observed an increased expression of MRC2 in a unique subtype of $CD45^+$ fibroblasts, commonly known as fibrocytes.[65] This novel immune population of hematopoietic-derived and fibroblast-like cells, presents characteristics of both macrophages and DCs and has been described in chronic inflammation, autoimmunity, fibrosis and scar formation.[67,71,76] With this in mind, we decided to characterize this immune population to better understand how its mannose-sensing properties impact the immunopathogenesis of HS. Therefore, we have characterized the expression of MRC2 in fibrocytes and its subsequent activation, in the presence of its ligand high-mannose *N*-glycans. Our results indicate that when in incubation with OVA-Man_9, fibrocytes display a significantly increased expression of MCR2, as well as a higher frequency of $MRC2^+$ fibrocytes. This may suggest the detection of mannose residues by fibrocytes via MCR2, with its subsequent overexpression, in a positive feedback mechanism. Given that, we can hypothesize that increased levels of mannosylated antigens in the skin of HS patients may promote fibrocytes' activation and recruitment towards inflamed tissues, being a potential driver of pro-inflammatory responses related to HS onset. Nevertheless, and although promising, these results require further validation.

Additionally, we have also observed a significantly higher frequency of MHC II^+ fibrocytes and a tendency for higher levels of CD86 co-stimulatory molecule. Importantly, both MHC II and CD86 markers are general characteristics of APCs, suggesting increased levels of antigen presentation by fibrocytes upon interaction with OVA-Man_9. Considering this, a co-culture of fibrocytes and T cells would be interesting to understand the importance of fibrocytes in the immunopathogenesis of HS, namely, to evaluate their antigen-presentation to naïve T cells that, ultimately, may activate B cells to produce anti-Man_9 antibodies. In fact, the antigen-presenting capacity of fibrocytes has been widely explored in a wide range of pathological conditions. In cancer, the blockage of PD-L1/PD-1 was demonstrated to increase the antigen-presenting capacity of fibrocytes, due to the higher expression of costimulatory CD86 and coinhibitory PD-L1, resulting in the activation of naïve T lymphocytes and a boosted antitumor immune response by $CD8^+$ T cells.[70,131] Chesney *et al.* [69] demonstrated that mouse fibrocytes also express the cell surface markers required for antigen presentation (MHC II, CD86, CD80), resembling the characteristics of human fibrocytes. Next, antigen-pulse (p24 and gp120 from HIV) mouse fibrocytes were injected into the inflamed site, being able to migrate into lymph nodes and specifically priming naïve T cells into $CD4^+$ T cells. Moreover, concerning autoimmunity, Cutolo *et al.* [132] have demonstrated that SSc patients display increased levels of circulating fibrocytes, in comparison to HC. Accordingly to our results, the same authors demonstrated that fibrocytes adopt a spindle-shaped morphology when cultured *In vitro*, together with the expression of MHC II and CD86. Then, the treatment of SSc

patients' fibrocytes with anti-CTLA4-Ig resulted in a lower expression of CD86 and a subsequent lower antigen-presenting rate to naïve T cells. Altogether, these results pinpoint the importance of fibrocytes in bridging innate and adaptive immune responses, being implicated in inflammation, cancer and autoimmunity.

Furthermore, we decided to analyse the cytokine profile of the fibrocytes incubated with glycoantigens, to determine whether the interaction with mannosylated structures would induce a more pro-inflammatory phenotype of fibrocytes, that could contribute to the inflammatory responses of HS and, ultimately, contribute to the B cell response. Unfortunately, our results are not very enlightening, since the concentration of IL-1β, TNF, IL-8, IL-6, IL-10 and IL-12p70 is similar in all the tested conditions (Basal, Ova-Man_9 and OVA-diGlcNAc). Nevertheless, since the cytokine profile was analysed in the supernatant of 72 hours incubation with glycoantigens, we can hypothesize that fibrocytes may require more time to release cytokines and that some differences in the cytokine profile may be identified in a longer period of incubation. Although promising, this analysis is still preliminary, and the fact that fibrocytes are not an isolated population, but enriched *In vitro*, may bias our results as we need to consider the influence of other immune cells that may also contribute to the pool of cytokines in the supernatant. Nevertheless, the characterization of fibrocytes should also be performed with PBMCs from HS patients, to compare the levels of circulating fibrocytes in HC and HS patients and to characterize the expression of MRC2, CD86 and MHC II in the context of disease. Similarly to Cutolo *et al.* [132], a co-culture of fibrocytes and anti-Man_9 antibodies from HS patients would also be valuable, to check if anti-Man_9 IgGs can interact with fibrocytes and in some way promote their activation (CD86 and MHCII), contribute to the inflammatory responses by the production of pro-inflammatory cytokines, or even enhance the expression of MRC2, essential to recognize the abnormal glycosignature of HS patients.

Finally, we used a glycoengineered mice model susceptible to colitis (VT1), in which the abolishment of *Mgat1* expression in the villin-positive cells results in the abnormal expression of high-mannose *N*-glycans in the gut. We used this mice model to better establish the gut-skin axis that we propose and to check whether the exposure of mannose-enriched *N*-glycans in the gut would promote alterations in the skin (e.g., glycome, HS-like skin lesions, inflammation), either mediated by circulating fibrocytes and/or ASCA-IgGs. In our *In vivo* data we observed that *VT1*$^{-/-}$ aging mice revealed significantly increased levels of serological ASCA-IgGs. Interestingly, the VT1 mice model concomitantly presents an abnormal glycosignature in the gut together with increased levels of ASCA-IgGs, possibly recapitulating the context of IBD, in which a gut glycome alteration associated with inflammation has been described, together with the prevalence of ASCA-IgGs as a predictive biomarker.[32,97,133] Additionally, we characterized the skin from *VT1*$^{+/+}$ and *VT1*$^{-/-}$ aging mice by histology and FACS analysis, including the assessment of the immune population ($CD45^+$), glycome ($CD45^-$) and morphology of the skin. Regarding the histological analysis, we did not observe any significant alterations between WT and KO aging mice, although the presence of immune cells in the KO mice is slightly increased. Nevertheless, both *VT1*$^{+/+}$ and

VT1$^{-/-}$ mice present a healthy skin phenotype without the morphological lesions typical of HS. Concerning our cytometry data, the epithelial glycosignature of the skin was assessed by GNA and SNA lectins, although no differences were detected in both WT and KO mice, as this conditional KO mice display an altered gut glycoprofile. This evidence, together with the histological data, suggests that the abnormal exposure of mannosylated epitopes in the gut is not sufficient to induce inflammation in the skin and alterations in its morphology, highlighting the need for a second glyco-hit to establish the axis.

Additionally, we have characterized the skin immune population (CD45^{+}) by FACS analysis, including CD4^{+}, CD8^{+}, γδ T cells, B cells and fibrocytes. Our results suggest that the KO mice tend to display increased levels of CD45, as described in the histological analysis, although not significantly. Then, the percentage of CD4^{+}, CD8^{+}, γδ T cells and B cells is similar in both *VT1*$^{+/+}$ and *VT1*$^{-/-}$ mice, and no significant alterations were detected. However, the only significant alteration in the skin immune population relates to the percentage of fibrocytes, which is markedly reduced in the skin of KO mice. Taking into consideration that fibrocytes are equipped with mannose-sensing receptors and, given the abnormal accumulation of mannose-enriched *N*-glycans in the gut, we can hypothesize that fibrocytes are being recruited to the gut due to mannose stimuli. In fact, Sazuka *et al.* [134] have demonstrated a prevalence of fibrocytes in tissue samples from CD patients together with significantly increased levels of circulating fibrocytes, in comparison with HC. Then, the authors suggest the recruitment of fibrocytes during the inflammatory phase of CD, contributing to inflammation by the release of TNF-α, which ultimately may differentiate into fibroblasts leading to fibrosis. Similar evidence was also described by Ueno *et al.*, [135] where the number of fibrocytes was significantly increased in CD patients displaying fibrosis of the bowel, which results in the development of strictures and a more complicated disease phenotype. Importantly, the increased prevalence of circulating fibrocytes was found to correlate with an increased need for surgery. Concordantly, Mukherjee *et al.* [136] have analysed full-thickness bowel scRNAseq data, demonstrating fibroblast heterogeneity in both mucosa and submucosa layers, revealing the important role fibroblast, and their fibrocytes precursors, in the pathogenesis of strictured CD. Afterwards, significantly increased levels of CCR2^{+}-monocyte-derived fibrocytes were found in azoxymethane (AOM)/dextran sodium sulphate (DSS) induced-colitis mice model, after the thirst cycle of DSS. In fact, fibrocytes were described to migrate into the inflamed colon during chronic DSS cycles, directly contributing to further colonic inflammation and fibrosis, by the production of TIMP-1 which inhibits collagen degradation.[137] Altogether, this evidence supports the role of fibrocytes in CD, therefore sustaining our hypothesis that fibrocytes may be located in the gut of *VT1*$^{-/-}$ aging mice, due to the immunogenic capacity of mannose-enriched *N*-glycans, although further studies are required to better understand the role of fibrocytes in this mouse model. Importantly, the role of fibrocytes should not only be checked for HS but also for CD, evaluating its potential in driving a gut-skin axis, together with anti-Man$_9$ antibodies, as previously mentioned.

The VT1 mice model could be an interesting model to characterize the gut-skin axis that we propose, although further studies are necessary to dissect this mice model in more detail. In fact, and to better characterize this axis, the abnormal mannose trigger should be present in both skin and colon. Thus, an interesting approach would be to chemically treat the skin of these mice with Kifunensin glycan inhibitor, which impairs the ER α-mannosidase from the *N*-glycosylation pathway, leading to the accumulation of $Man_9GlcNAc_2$ structures.

5. CONCLUSION & FUTURE PERSPECTIVES

The results obtained in this thesis constitute a valuable tool to better understand the immunopathogenesis of HS and CD, as well as to characterize the immunological factors responsible for mediating a gut-skin axis and, subsequently, the association between both autoimmune illnesses. Our findings support the existence of a glycan switch event in the transition from health to an autoimmune phenotype, essentially characterized by the accumulation of high-mannose *N*-glycans in HS and CD, pinpointing a common glycan-based mechanism between both disorders. Additionally, we have contributed to clarify the role of high levels of anti-Man_9 in HS and CD, which may resemble ASCA-IgGs, suggesting their role in mediating the gut-skin axis that we proposed. In addition to anti-Man_9 antibodies, our evidence indicates the role of circulatory fibrocytes in mediating a gut-skin axis, due to their mannose-sensing properties, which may mediate the recognition of altered glycosignature in both skin and colon. Moreover, we have demonstrated the potential role of the VT1 mice model in studying the gut-skin axis that we proposed, although further studies are required to better characterize this glycoengineered mice model.

As a future perspective, we aim to validate these preliminary results in a validation cohort to correlate the abnormal glycosignature with the clinical variables of both HS and CD. To better understand the glycan mimicry between Man_9 and mannan structures, essential for the loss of immune tolerance, it would be interesting to functionalize mannan to a primary amine and then conjugate it to OVA. In fact, the OVA-mannan would mimic the glycoepitopes of ASCA-IgGs, allowing us to better understand its role in the immunopathogenesis of HS and CD. Similar to the western blot assay, we also intend to follow the same approach with skin proteins from HC and HS patients and anti-Man_9 antibodies from HC and CD patients. To validate the cross-reactivity of HS antibodies and CD proteins, an immunohistochemistry assay with FFPE skin samples from HS and antibodies from CD patients would be also valuable to better characterize the gut-skin axis. Regarding the fibrocytes, further studies are required to better characterize this immune population in the context of disease and to evaluate their potential in mediating the gut-skin axis. Concerning the VT1 mouse model, an immunofluorescence assay would be important to confirm the presence of fibrocytes in the gut. Finally, the abnormal glycosylation trigger in this mice model is required in both skin and gut, to better understand the axis between the two, and to fulfil this we intend to treat the skin of these mice with Kifunensin, as previously mentioned.

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7. APPENDIX

7.1. Appendix 1

ST6Gal1 SYBR Green primers sequence:

Forward 5'- AGCGCTTCCTCAAAGACAGT -3'

Reverse 5'- TCCGGATTCTGGTACCACTTTG -3'

GAPDH SYBR Green primers sequence:

Forward 5'- CAAATTCCATGGCACCGTCAA-3'

Reverse 5'- ATGGTGGTGAAGACGCCAGTG -3'

7.2. Appendix 2

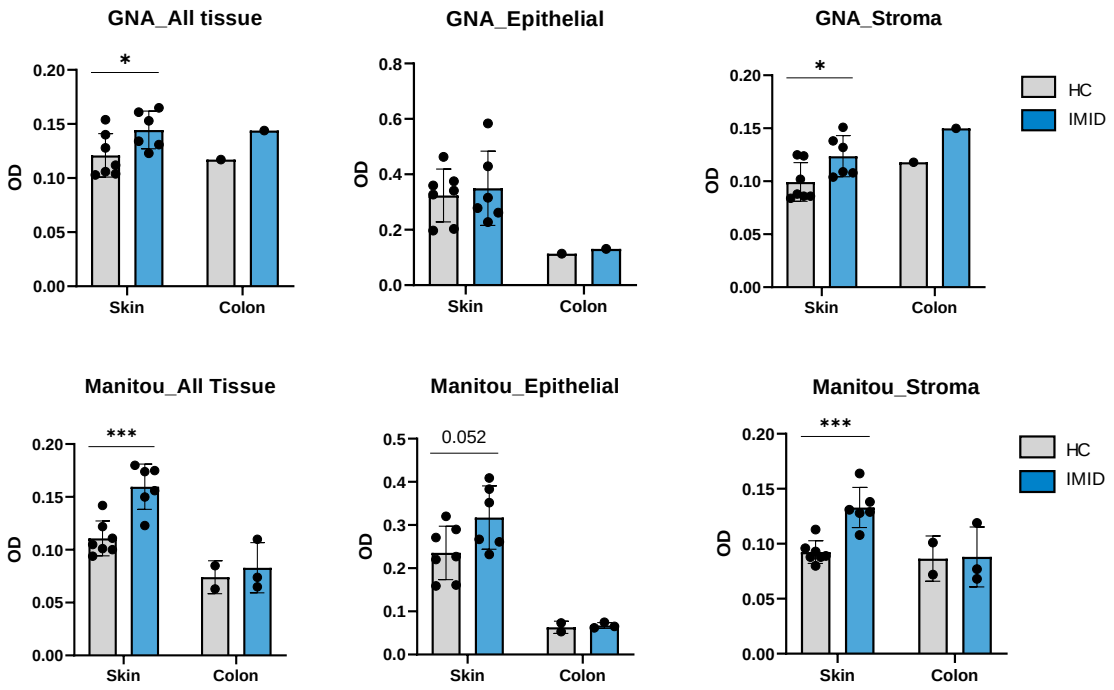

Figure 7.1 | Quantitative quantification of lectin reactivity in GNA and Mannitou histochemistry. Schematic representation of the quantitative evaluation of GNA and Mannitou histochemistry from HC (skin n=7; colon n=2), HS (n=6), and CD (n=3) patients. The evaluation was performed on ImageJ v8 and in three subcategories: all tissue, epithelial and stromal compartments. Comparing HS patients to the HC, both GNA and Mannitou expression is statistically higher when evaluating skin all tissue and stromal components, although these differences are not so striking for the CD cohort. Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: *p ≤ 0.05, ***p ≤ 0.001.

7.3. Appendix 3

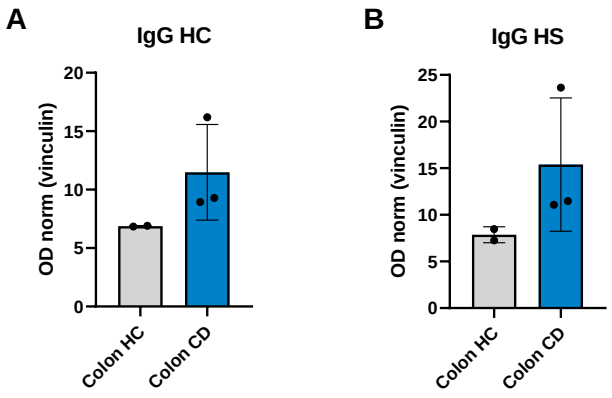

Figure 7.2 | Quantification of the western blot assay with colon HC and CD TCL with IgGs from HC (A) and HS (B). Statistical significance was assessed by the non-parametric Mann-Whitney T-test: not significant.

7.4. Appendix 4

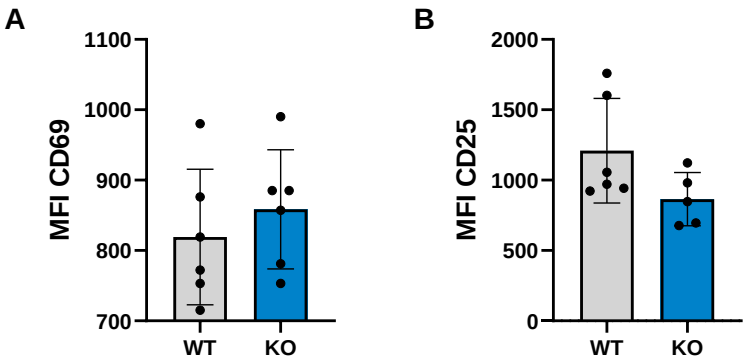

Figure 7.3 | Activation of γδ T cells in VT1 aging mice skin characterization. Median fluorescence intensity (MFI) of CD69 (A) and CD25 (B) activation markers of γδ T, which correspond to early and late activation markers, respectively. No significant alterations were observed. Statistical significance was assessed by the parametric Shapiro-Wilk test or non-parametric Mann-Whitney T-test: not significant.