# A study of autophagy gene expression in autoimmune Addison's disease

Master's thesis in Molecular Biology

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May 2022

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# Acknowledgment

First and foremost, the greatest of thanks and nods of the head to my supervisor André Sulen, for guiding me through the complex and mind-bending world of immunity research. The number of times where you had to explain or show the same thing to me more than once would have exceeded the patience of many scientists who have reached a higher level in research, but not even once did you make me feel annoying, condescended or stupid. To not let one's "intelligence" corrupt one's ego is a true virtue, and I appreciate you for holding on to it.

The same applies to the rest of the group. Special thanks to Lars for a great sense of humour, a wide field of fun facts, and for updating me on what my brother is doing when I haven't checked in for weeks. Anette and Bergithe for smiling so often and sharing their warm presence. Shahin, for making me not feel like the only one who is confused about existing. My co-students Emina, Adrianna and Ifunanya, for partaking on this journey with me.

My parents. Even though the choice of studying molecular biology came from within, the belief that I could take on such a task was passed on from you.

Luisa and Nøstet Badeklubb. For sharing havens to just be, leaving behind the future and past, and diving into the love of the present.

Markus, for giving me both physical and mental space in the period of writing my thesis. Nourishing it without me even noticing, before looking to my right and noticing a baby snail in a water vase.

Regjeringen and lånekassen, for supporting a lifestyle grounded in the yearning to learn.

And finally, myself. For not being hard on myself for too long upon mistakes. For holding on to my way of being, following intuition (even when it wants scary things), and always believing, even if things don't go as planned, that it's going to be alright.

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May 2022

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## Abstract

Autoimmune Addison's disease (AAD) is characterized by the deficiency of vital hormones produced by the adrenal glands as a result of their destruction by autoimmune cells. Reported to be increasing in its prevalence, with western Norway showing the highest amount of autoimmune adrenal insufficiency in the world, the exploration of new pathways leading to its onset is crucial for understanding the pathogenesis of this disease. Such a pathway could be the "devouring" mechanism of autophagy, which has been shown to be involved in a plethora of immune cell functions, including their maintenance, regulation and activation. As AAD is characterized by a dysregulated immune system, we hypothesized that autophagy was dysregulated as well, as has been shown to be the case in numerous other autoimmune-related diseases.

To indulge on this hitherto unexplored connection, the expression of autophagy genes was compared in healthy controls and patients with AAD. This was granted via access to the national registry and biobank of autoimmune diseases (ROAS), allowing for the isolation of RNA from blood of patients with AAD. Some of these had also been sequenced for their presence of the risk allele of protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), which has been shown to be reported in a higher frequency in patients with AAD, allowing for the connection of this allele to autophagy to be explored as well. Further, the abundance of neutrophils and monocytes in AAD patients were compared to healthy controls to see whether any alterations in autophagy gene expression was a consequence of their perturbed abundance of these cells in whole blood. Finally, the human monocytic cell line THP-1 was used to further explore the role of *PTPN22* in autophagy in vitro, by knocking down the expression of *PTPN22* via siRNA transfection.

The resulting study found several autophagy genes to be altered in whole blood of AAD patients compared to control, namely *ULK1*, *ULK2*, *ATG9A*, *ATG12*, and *GABARAP*. This was found to be independent of the abundance of monocytes and neutrophils. The risk allele of *PTPN22* was found to be associated with the expression of *ATG12*, *GABARAP* and *ATG5* in whole blood of AAD patients, and *PTPN22* association with *ATG5* was further confirmed in *PTPN22*$^{KD}$ THP-1 cells in vitro. Whether these associations relate to the products of these genes requires further proteomic studies, and larger sample sizes for the gene expression assessments derived from the whole blood of patients.

## Abbreviations

| | |
|---|---|
| AAD | Addison's autoimmune disease |
| AIRE | Autoimmune regulator |
| APC | Antigen presenting cell |
| BCR | B cell receptor |
| BRT | Blood RNA Tube |
| CD | Cluster of differentiation |
| C(X) | Complement component (X) |
| cDNA | Complementary deoxyribonucleic acid |
| cTEC | Cortical thymic epithelial cell |
| CTLA4 | Cytotoxic T-Lymphocyte Associated Protein 4 |
| DAMP | Damage-associated molecular pattern |
| DC | Dendritic cell |
| DEPC | Diethyl pyrocarbonate |
| DNA | Deoxyribonucleic acid |
| ds | Double stranded |
| FACS | Fluorescence activated cell sorting |
| FBS | Fetal Bovine Serum |
| FCS | Forward light scattering |
| FOXP3 | Forkhead box protein 3 |
| GWAS | Genome wide association study |
| h | Hour |
| Ig | Immunoglobulin |
| IL | Interleukin |
| KD | Knockdown |
| M1 | Classically activated macrophage |
| M2 | Alternatively activated macrophage |
| MHC | Major histocompatibility complex |
| mL | Millilitre |
| MP | Macrophage |
| mTEC | Medullary thymic epithelial cell |
| min | Minutes |
| ng | nanogram |

| | |
|---|---|
| NLR | NOD-like receptor |
| NOD | Nucleotide-binding oligomerization domain |
| PAMP | Pathogen-associated molecular pattern |
| PCR | Polymerase chain reaction |
| PE | Phosphatidylethanolamine |
| PRR | Pattern-recognition receptor |
| PTPN22 | Protein tyrosine phosphatase non-receptor type 22 |
| qPCR | Quantitative polymerase chain reaction |
| Ref | Reference |
| RNA | Ribonucleic acid |
| RNFW | RNase-free water |
| ROAS | Registry for organ-specific autoimmune diseases |
| s | Seconds |
| SSC | Side light scattering |
| TCR | T-cell receptor |
| TGF-β | Transforming growth factor β |
| $T_h$ cell | T helper cell |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| Treg | Regulatory T-cell |
| µL | microlitre |
| µm | micrometer |

# 1. Introduction

## 1.1 The immune system

The collection of cells, chemicals and processes that free their host organism from pathogens and bodies of disinterest is referred to as the immune system, and their coordinated response towards infectious agents is an immune response[1]. It is an essential component prevalent in nearly all life forms, with corresponding systems present in everything from bacteria and archaea, to ancient plants and animals [2][3], as well as their modern descendants. In humans and other advanced vertebrates, these defence mechanisms have evolved into two cooperative parts: the innate and adaptive (Figure 1.1). Innate responses are preconfigured and rapidly block infectious agents in the same manner each time they are encountered, whilst adaptive responses are tailored upon repeated exposure to the given infection, which is why this system is also referred to as acquired immunity [1][4].

Phylogenetically, innate immunity is older, while the adaptive system evolved as a more specialized and powerful tool later, gaining the ability to co-adapt to the many mutations and variations of ever evolving pathogens. Its network of action is the primary and secondary lymphoid organs: the bone marrow and thymus, and the lymph nodes, spleen, and various mucous membrane layers such as the tonsils[5]. Utilizing cytokines, messenger molecules which orchestrate and balance immune responses between humoral and cell-based, innate and adaptive systems secure both extracellular and intracellular spaces of their host. Extracellular, by inducing what is called a humoral response through antibodies (Abs) and complement proteins circulating the humours/body fluids, and intracellular, by utilizing cell-based mechanisms such as T-cell mediated apoptosis and macrophage mediated phagocytosis – the literal devouring of particles ($\geq 0.5$ μm). Indeed, the immune system is a collaborative effort between distinct, but complementing subsets, enabling and enhancing one another in the common goal of eradicating invading pathogens[6][7][8][9].

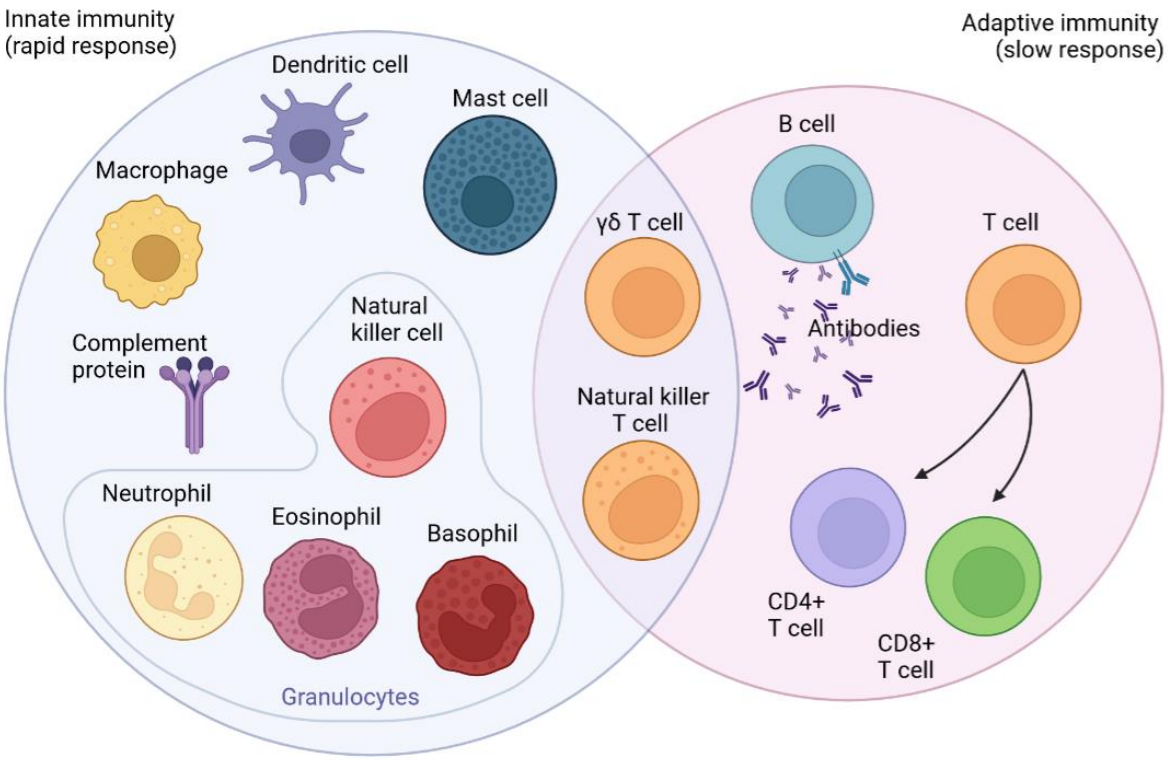


**Figure 1.1: Cells of the immune system.** All descendants of the multipotent hematopoietic stem cells (HSCs) of the bone marrow, transition through haematopoiesis allows for the differentiation of distinct cell types with specialized functions. Innate immunity comprises granulocytes (basophils, eosinophils and neutrophils) as well as mast cells, dendritic cells, macrophages and natural killer (NK) cells, whilst adaptive immunity comprises T and B cells which are able to further differentiate into respective subclasses. The intersectional (purple) cells bridge the two systems together in that they are of T cell lineage but behave in a manner typical to the innate immune system. Modified from Dranoff [10] using BioRender.

## 1.2 Innate immunity

Also called the natural, or native immunity, this defence strategy consists of dendritic cells (DCs), monocytes, macrophages, granulocytes, innate lymphoid cells (ILCs) and natural killer (NK) T cells[11][4], as well as the tissues forming the interface between an organism and its environment, such as skin, pulmonary and gut epithelial cells. In addition to cellular responses, innate immunity also utilizes non-cellular methods to tackle pathogens and bodies of disinterest, methods ranging from simple structural barriers such as the stratum corneum covering the outer epidermis with several layers of dead protective tissues, to complex cascades of protein precursors which ultimately aid the adaptive system as well [6][12].

Activation of the innate immune system is carried out through the recognition of conserved motifs in pathogens and indicators of cell stress or death, referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively [8]. These danger signals

are detected by pattern-recognition receptors (PRRs), a key property of innate immune cells subdivided in classes such as transmembrane toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, further shortened NLRs). These receptors in turn trigger an immune response, resulting in the release of pro-inflammatory cytokines which encourage inflammation: localized redness, swelling, heat and pain as a result of leukocyte and fluid movement through increasingly permeable capillaries to the site of infection [13]. Humoral activation takes place in parallel via the activation of one of the complement pathways, such as the classical where complement component 1 (C1) binds directly to the pathogen's surface initiating the complement system [14]. Together, cell-based and humoral components of innate immunity orchestrate a range of defence mechanisms, such as coagulation, recruitment of phagocytic neutrophils and monocytes/macrophages, opsonization (tagging pathogens for phagocytosis), induction of apoptosis, and also the recruitment and activation of cells of the adaptive immune system [8][13][15][16].

### 1.2.1 Neutrophils

Focusing on human phagocytes, which have their names derived from the Greek *phagein* meaning "devour", and *kutos* meaning "hollow vessel" (referring to the cell), their anti-infectious properties are indeed a vital part in dismantling disease as they engulf and degrade pathogens and foreign particles. Neutrophils are the most abundant phagocyte numbering 4000 to 10,000 per millilitre (mL) blood, a number that may rise to 20,000 per mL in the event of pro-inflammatory cytokine release which stimulate hematopoietic stem cells in the bone marrow, causing proliferation and maturation of neutrophil precursors. Being the first cell type to respond to most infections, neutrophils are the dominant cells of acute inflammation, responding particularly to bacterial and fungal infections which they may digest and destroy. They are also recruited to sites of tissue damage which are not exposed to infection, where they may clear cell debris. Neutrophils only live for a few hours in tissues and do not provide prolonged defence; especially after having initiated phagocytosis, where they are digested themselves by macrophages[17][1].

### 1.2.2 Monocytes

A progenitor for both macrophages (MPs) and dendritic cells (DCs), monocytes number 0.3-0.9 million per mL blood in humans[18], being less abundant than neutrophils although living for much longer[1][19]. Indeed, the life cycle of monocytes is more complex compared to their partners in crime, following differentiation abilities which upon cytokine stimulation may generate antigen-

presenting cells (APCs), phagocytic, and also immunomodulating cells, fitting to their role as second responders arriving at infection sites after the neutrophils. Although the functions of antigen presentation and inflammatory/anti-inflammatory phagocytosis are the main subjects of DCs and MPs respectively, they are not reserved to each part as both differentiated outcomes of monocytes may serve these roles. This taskforce is added upon by the classical, non-classical and intermediate subsets of monocytes which circulate the blood as still-uncommitted cells, as they themselves have been shown to possess phagocytic, antigen-presenting and inflammatory, and phagocytic and inflammatory properties, respectively[20].

Historically, monocytes have been viewed as transitional cells that adapt to repopulate either DC or MP populations indiscriminately, and it took decades between postulation and determination of monocyte heterogeneity. Ultimately, they were divided by the surface receptors which the different subsets have shown to exhibit [21]. The majority of monocytes have high levels of lipopolysaccharide (LPS) coreceptor cluster of differentiation (CD) 14 and low levels of expression for CD16: these are the classical monocytes ($CD14^{++}$; $CD16^{-}$). Intermediate monocytes ($CD14^{+}$; $CD16^{+}$) show lower CD14 and more CD16, and non-classical monocytes ($CD14^{-}$; $CD16^{+}$) show the least amounts CD14 and high amounts CD16. Although there are more receptors upon which antibodies could bind and identify subsets such as chemokine receptor type 2 and 5, the identification of monocyte subsets through CD14 and CD16 receptors are well established and therefore the ones most frequently used[22][23].

### 1.2.3 Macrophages

While monocytes effectively perform immune functions circulating in the blood, their migration into tissues causes a progressive alteration in their phenotype, as local growth factors induce their transition to resident macrophages [24]. In contrast to neutrophiles, macrophages are integral components of tissues which are recruited continuously and not just upon pro-inflammatory cytokine stimulation, as they contribute to organ development and the maintenance of homeostasis. They also serve several important roles in host defence, with arms of both humoral and cell-mediated immunity: producing cytokines that regulate inflammation, ingesting and destroying microbes through phagocytosis, and cross-presenting the antigens of said microbes to cells of the adaptive immune system. Tissue resident macrophages are extremely heterogeneous [25], with divergent phenotypes and functions dependent on both the microenvironments of the distinct tissue in which they reside but also on their origin. Indeed, not all macrophages are derived from monocytes: populations derived from the embryonic yolk sac are co-maintained in circulation, although through self-renewal [26][27].

The activation pathway of these “big eaters” is grouped into two categories: M1 and M2. Also called classical macrophage activation, M1 macrophages are activated by the invasion of pathogens, induced

by PAMPs, DAMPs and inflammatory cytokines to trigger inflammation and destroy danger. Conversely, alternative macrophage activation M2 occurs in the absence of strong TLR stimulation and is responsible for healing the aftermath of pathogen invasion as well as downregulating inflammation through anti-inflammatory cytokines. It is considered that embryonic-derived macrophages play the strongest role in tissue homeostasis à la M2, whilst monocyte-derived macrophages play the innate immune responses of M1[1][27].

## 1.3 Adaptive immunity

Emerging only 500 million years ago as a second line of defence in vertebrates, adaptive immunity provides flexibility and memory to their host's immune system through T and B cells. Great hallmarks in evolution; adaptive immune cells undergo somatic recombination of receptor genes [28], a mechanism which result in an almost limitless catalogue of unique and novel antigen receptors, expanding upon the fixed repertoire of innate immunity and enabling the potential to recognize nearly all pathogens. The adaptive immune cells which encounter antigen are clonally expanded, and thus persist a population which declines over time in their host organism, allowing for rapid and specific responses to reinfection [29][30][31]. Both T and B cells develop and mature in the primary lymphoid organs: although both arise in the bone marrow, only the latter mature there; precursor T cells must traverse to the thymus in order to mature [32].

## 1.4 B cells

Developing from hematopoietic stem cells (HSCs) in the bone marrow, B cells are subjected to positive and negative selection in their maturation, ensuring the maintenance of central tolerance. This process involves transmembranal B cell receptors (BCRs), consisting of a signal transduction moiety and an immunoglobulin (Ig), key protein complexes which regulate the B cell's further development and activation which may ultimately lead to differentiation into antibody-secreting effectors cells known as plasma cells, as well as memory B cells containing BCRs which recognize the same antigen that activated their parent cell[33][34]. Antibodies are encoded by heavy and light Ig genes and form the basis of humoral immunity, which is essential in defending against extracellular microbes and toxins. Successful antibody secretion can induce actions such as neutralisation of bacteria or virions through blockage of surface proteins, agglutination and precipitation of pathogens which encourage phagocytosis, as well as complement activation and its corresponding defence mechanisms [1][35].

The activation of B cells and the following humoral response initiates in secondary lymphoid organs such as the spleen and lymph nodes, where B cells migrate towards after maturation [1]. Here, the B

cell must encounter an antigen which its BCRs recognize, to cause a reversible noncovalent interaction with affinity which varies upon whether it is the first time the antigen has been encountered (a primary Ab response incited by a naïve B cell), or if it has been encountered before (a secondary Ab response incited by a memory B cell), in which case a faster and greater response with higher amounts of antibodies are produced [31][33][36].

While many non-protein antigens can stimulate antibody production by themselves, antigens of foreign proteins require second signals provided by T helper ($T_h$) cells. Known as T cell-dependent (TD) activation, the mechanism involves a BCR binding a TD antigen which is engulfed into the B cell via endocytosis. The antigen is processed and then presented again as peptide pieces on the B cell's surface by major histocompatibility complex (MHC) II proteins, effectively making the lymphocyte an antigen-presenting cell (APC)[34][6]. The peptide-MHC-II complex is recognizable by the antigen-specific T cell receptor (TCR) of $T_h$ cells, which in binding induces the $T_h$ cell to express surface proteins such as CD40L, IL-4 and IL-21. These may thus bind to the B cell's surface and function as co-stimulatory signals and induce further activation and ultimately proliferation, underlining the important and complex dialog between B and T cells in determination of the ultimate immune response [36][37][38][39].

### 1.4.1 T cells

In contrast to B cells, T cells need to emigrate from the bone marrow to the thymus in order to mature. Here the precursor T cells undergo sequential steps defined by their presence or absence of cluster of differentiation (CD) 4 and 8, co-receptors for the T cell receptor (TCR) whose own development ultimately decides which CD expression is maintained and which is discarded[40][41]. This lineage decision, influenced by whether the lymphocyte's TCR recognize class I or II MHC molecules in complex with self-peptide, respectively determines the T cell to be deemed a $CD8^+$ "killer" cell or a $CD4^+$ "helper" cell, which together shape the function of immune-mediated cell death. An essential part of the immune system, cytotoxic $CD8^+$ T cells are able to directly kill cancer cells, infected cells, or cells damaged in other ways, while $CD4^+$ $T_h$ cell induce a larger immune response in co-operation with other immune cells[42][43].

Leaving the thymus programmed for one specific antigen, $CD8^+$ and $CD4^+$ T cells circulate the blood and lymph until their TCR recognize their cognate antigen on MCH I or II molecules, respectively, bound on the surface of an APC. This triggers initial activation of the T cells, causing the $CD8^+$ or $CD4^+$ molecules to bind the MHC complex of the APC and stabilize the whole structure [44]. While in binding, both $T_h$ cells and cytotoxic T cells require additional second signal for further activation. In the case of the $T_h$ cell, such a signal is provided by CD28, a protein on the T cell which binds to CD80

or CD86 on the APC thus initiating T cell proliferation. Further activation is then received through cytokines which decide the effector subtype the $T_h$ cell differentiates into, with options such as Th1 (driven by cytokine IL-12), Th2 (driven by IL-4) or IL-17 (driven by IL-6 and IL-23), each hosting a specialized immune response[45][46][47][48].

The close interaction between T cells and host cells in regard to pathogen treatment carries the potential to function as double-edged sword, with the possibility to do enormous damage to healthy tissues in the events of misdirection; that being T cells responding to self-antigens as opposed to foreign antigens. Fortunately, thymic selection and peripheral regulation includes mechanisms shaping self-tolerance through various molecular checkpoints as well as regulatory T cells (Tregs) suppressing the immune response. However, self-tolerance may be escaped, which gives rise to the unfortunate events of autoimmune diseases driven through autoimmune reactions[49].

### 1.4.2 Central T cell tolerance

Positive selection mechanisms of T cell maturation have been mentioned in the shape of TCR binding to a thymic MHC molecule in complex with a self-peptide, however, this mechanism is affinity-dependent, and may also result in clonal deletion, death by neglect, or the generation of $CD4^+$ regulatory T cells known as Tregs (Figure 1.2). Only T cells with TCR binding weakly to self-peptide-MHC complex are positively selected for differentiation into $CD8^+$ or $CD4^+$ lymphocytes; non-binding T cells are neglected, while strongly binding T cells are subjected to clonal deletion through apoptosis but may also be inducted the transcription factor forkhead box protein 3 (Foxp3) and differentiate into Tregs. Clonal deletion can be averted through undergoing secondary gene rearrangement at the TCRα loci, a concept known as receptor editing which eventually changes the TCRs affinity for the self-peptide-MHC [1][49][50][51].

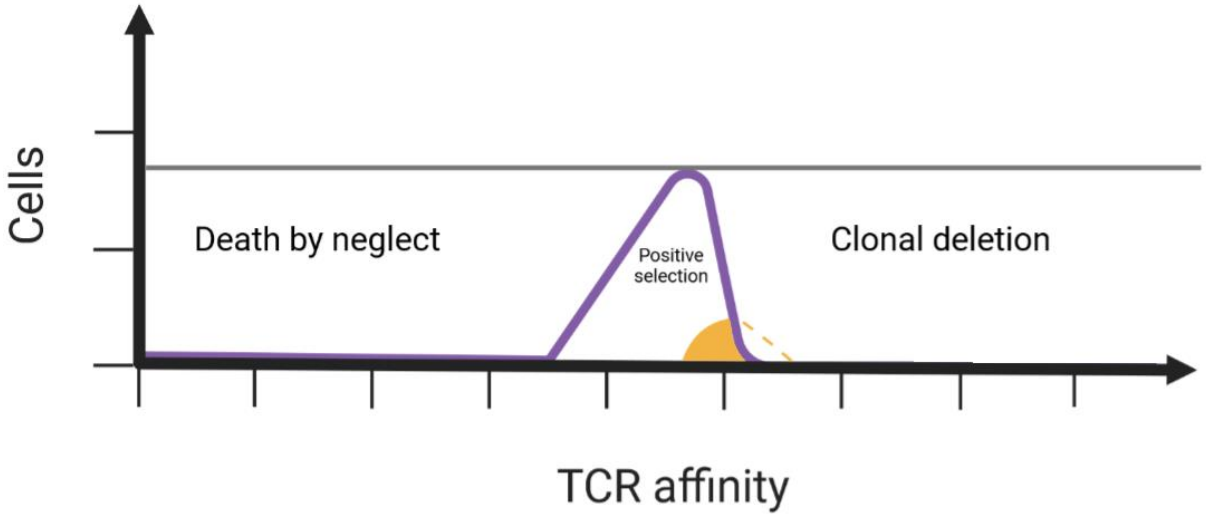


**Figure 1.2. Only a small fraction of T cells is allowed to mature.** Depending on their TCRs affinity for self-peptide-MHC, developing T cells in the thymus are either negatively regulated by neglect or clonal deletion, or positively selected to differentiate to CD8+ or CD4+ lymphocytes. TCRs of regulatory T cells (Treg) (yellow) have the highest affinity for self-peptide-MHC; some (dotted line) require rescuing by cytokine signalling or receptor editing in order to avoid deletion. Modified from Xing and Hogquist [49] by using BioRender and Paint.

Shaping the foundation of central tolerance in T cells, these mechanisms take place in the thymus' cortex, medulla, or their junction. Positive selection takes place in the cortex with the help of cortical thymic epithelial cells of the cortex (cTECs), while negative selection takes mostly place in cortico-medullary junctions and the medulla with the help of medullary thymic epithelial cells (mTECs), although may also occur in the cortex. mTECs play a vital role in the introduction of self-proteins to developing T cells, largely in part to their autoimmune regulator (AIRE) protein [49][52]. Functioning as a transcription factor, AIRE orchestrates the gene expression of tissue-specific self-antigens (TSAs) on mTECs, non-local proteins which are not normally found in the thymus but are present in other organs, thereby driving a negative selection on developing T cells which respond to these[53].

### 1.4.3 Peripheral T cell tolerance

Despite valiant efforts, not all self-reacting T-cells are contained in the thymus. Therefore, peripheral-tolerance mechanisms exist, ensuring the suppression or elimination of self-reactive lymphocytes which have escaped central tolerance. These control mechanisms include quiescence and ignorance, anergy, clonal deletion, and their conversion to Tregs which may further suppress the effector functions of conventional lymphocytes in the periphery.

#### 1.4.3.1 Quiescence and ignorance

Upon entering circulation from the thymus, naïve T cells are in a quiescent state, meaning that they are in the G0 stage of the cell cycle with low metabolic, transcriptional, and translational activities. Quiescence allows for T cells to remain alive and circulating in the host organism without being activated by tonic signalling. Quiescence exit begins when the T cell is stimulated by antigen exposure and co-stimulation, upon which proliferation, survival, and effector differentiation mechanisms are induced[54].

Ignorance is another aspect of peripheral tolerance, which leads to self-reactive T cells never activating due to failure of binding. This involves intrinsic and extrinsic mechanisms: intrinsic, in the sense that the cell's TCR affinity the antigen is too low to elicit a T cell response, and extrinsic, in that the antigen in question is either present in another anatomical position of its host organism or exists in too low concentrations to ever meet the self-reacting T cell [55].

#### 1.4.3.2 Anergy

Anergy in T cells refer to a long-term hyporesponsive state where they are functionally inactivated. As mentioned earlier (1.3.2) full activation of T cells require a second signal which is provided by APCs, and it is indeed the failure of this co-signalling which induces anergy. Successful activation involves the nuclear factor of activated T cells (NFAT), which is induced by TCR stimulation, forming a complex with activator protein 1 (AP-1), which is induced by co-stimulatory molecules. Without the latter transcription factor, NFAT will homodimerize with itself instead, and become a transcription factor which induces anergy in the lymphocyte[56][57].

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is also involved in the functional inactivation of T cells, as it binds to CD80/CD86 with greater affinity than CD28 does and acts as its competitive inhibitor. Since CD28 co-stimulation is necessary for further activation of T cells, the mechanisms of CTLA4 effectively functions as a negative signal which prevents cell cycle progression [58][59].

#### 1.4.3.3 Deletion

The recognition of self-antigens may trigger pathways of apoptosis resulting in the deletion of the self-reactive lymphocyte. Two pathways remain the most likely, both triggered during APC-binding to the T cell. The first one is regarded to pro-apoptotic proteins which are induced upon antigen recognition: a normal immune response also expresses anti-apoptotic proteins induced by co-stimulation and growth factors, together which counteract these pro-apoptotic proteins. Thus, the absence of sufficient

co-stimulation results in various mitochondrial proteins leaking out and activating cytosolic enzymes (caspases) which induce apoptosis[55]. The second is regarded to tumour necrosis factor (TNF) superfamily members called death receptors which may be expressed upon the recognition of self-antigens. Of the death receptors one may regard the interaction of CD95 and its ligand CD95L as the most defined in regard to self-tolerance. The expression of CD95L following T cell activation has been regarded as lethal weapon used to eliminate target cells, as it initiates a proteolytic cascade following CD95 binding, recruiting and activating caspases 8 and 10 which further initiate the extrinsic apoptotic pathway[1][60][61][62].

#### 1.4.3.4 Immune suppression by Tregs

Suppressive and immunomodulatory functions of Tregs is crucial for the maintenance of immune homeostasis, which helps to prevent autoimmune disease and reduce potential damage induced by self-reacting lymphocytes. Tregs generated in the thymus have already been discussed, however, Tregs may also be generated in the periphery: termed adaptive, or peripeheral Tregs, they often carry similar phenotype and function as the ones generated in the thymus, termed natural Tregs, although use different mechanisms to fulfil their means. Indeed, while natural Tregs engage in suppression via direct cell-cell interactions, peripheral Tregs mediate their inhibitory activities by producing immunosuppressive cytokines, although cell-cell contact might still be necessary to initiate the suppressive cascade[50][63].

Now that we have described the principal mechanisms of self-tolerance, we consider the consequences of the failure of self-tolerance, namely, the development of autoimmunity.

## 1.5 Autoimmunity

Autoimmunity is defined as immune responses directed against the host organism's own healthy cells, tissues and other body normal constituents[1][64]. There are multiple pathways leading to its cause, and not all cases are pathogenic. In fact, low levels of autoimmunity are physiological and essential for lymphocyte selection and immune system homeostasis. Intermediate levels occur as minor tissue infiltration by circulating autoantibodies without clinical consequences and is not deemed pathological until organ injury and disease occurs[65]. Controlled levels of autoimmunity may even be induced to repair injuries and treat diseases such as Alzheimer's [66][67].

Based on the extent of tissues involved, autoimmune diseases are divided into two classes: organ-specific and systemic diseases. While organ-specific autoimmune disease only affects one organ, like

in the case of Addison's disease and Type I Diabetes (T1D), systemic diseases such as systemic lupus erythematosus (SLE) involve multiple organs by targeting ubiquitous substances like DNA. Effector molecules inducing these diseases may vary in their involvement of autoantibodies and cytotoxic $CD8^+$ T cells, but all instances require $CD4^+$ $T_h$ cells[1][65]. How they develop also varies, although three factors are at play: genes, immune system mediators, and the environment where the patient lives. Indeed a collaborative endeavour, genes confer the predisposition of autoimmune disease taking place, a dysregulated immune system offers the tools for executing pathological damage, and the environment delivers the final blow, with triggers that make autoimmunity clinically apparent[68].

Research on the subject of autoimmunity is of great importance as it affects approximately 8% of the population, a number which is seemingly rising. Women are more exposed than men, and the elderly have higher autoimmunity but lower prevalence of disease[69][70]. A large body of recent publications have revealed disturbances in the ecosystem of the microbiota, our symbiotic partners in maintaining physiology, longevity, metabolism, and immune system development. One cannot pinpoint a specific aetiology to the rise of autoimmunity, although the adverse effects of microbiota dysbiosis is not to be ignored[65].

## 1.6 Autoimmune Addison's disease

Also known as primary adrenal insufficiency, autoimmune Addison's disease (AAD) is the deficiency of glucocorticosteroids and mineral corticosteroids, caused by an autoimmune attack of the adrenal cortex which renders damaged adrenal glands unable to produce these hormones in sufficient amounts. Being essential regulators of water- and electrolyte homeostasis as well as energy balance, their deficiency leads to weakness, weight loss, anorexia, orthostatic hypotension due to dehydration, salt craving, hyperpigmentation, musculoskeletal and abdominal pain, nausea, vomiting and ultimately death. It is fortunately a rare disease, with treatment available in the shape of lifelong steroid supplementation replacing the deficient hormones. However, like most other autoimmune diseases, AAD's prevalence has been reported to be increasing[71], with western Norway having shown to yield the highest reporting of autoimmune adrenal insufficiency in the world [72].

AAD exists either as an isolated event or arises as part of a larger heterogenous group named autoimmune polyglandular syndromes (APSs), diseases which are characterized by autoimmune activity against more than one endocrine gland. While the latter branch of diseases often has known and specific genetic backgrounds such as *AIRE* gene mutations in APS-1, all cases of AAD show association to gene variants of the MHC. Certain alleles of the human leukocyte antigen (*HLA*), which display MHC-like molecules in humans, have been linked to the onset of AAD, indicating that T cells play an important role in these disorders (since the only known function of MHCs are in antigen

presentation to T cells)[71][73][74]. This link has been shown to be even greater among monozygotic (identical) twins than among dizygotic twins, proving the importance of genetics in the susceptibility of AAD[1].

Recently, the joint operation of the world's two largest AAD biobanks allowed for a genome-wide association study to take place, where the nine highest risk loci were identified. In addition to *HLA* and *AIRE*, loci shared with other autoimmune diseases in regard to lymphocyte function and development were *CTLA4*, broad complex-tramtrack-bric a brac and Cap'n'collar homology 2 (*BACH2*), and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*)[75], of which the latter will be in focus for our study. A negative regulator of TCR and BCR signalling, *PTPN22* has been referred to as the archetypal non-HLA autoimmunity gene, shown to be of great importance for the maintenance of homeostasis in the activation of its host cell. Some genetic variants of *PTPN22* in humans are associated with increased risk of autoimmune disease, as in the case of 620R->W which causes a gain-of-function in its role, suppressing TCR/BCR signalling and ultimately leading to an increased number of self-reactive T and B cells escaping central tolerance[76][77].

## 1.7 Autophagy and autoimmune disease

A key player to take into regard when one is discussing mechanisms of the immune system is autophagy. Greek for "self-eating", autophagy is an evolutionary conserved process by which cells target their own cellular organelles and long-lived proteins for degradation. Despite being termed and theorized upon since the early 60's, it took decades before substantial breakthrough was made on the many functions of autophagy, which is now known to range from the utilization of reserve proteins during nutrient starvation, to tissue reparation, programmed cell death and many more still being discovered[78][79][80][81][82]. Autophagy has roles in both innate and adaptive immune systems, aiding in the degradation of bacterial pathogens, enhancing their delivery to lysosomes and therefore helping the delivery of pathogen-related peptides to the MHC[83]. Indeed, learnings of the past years in regard to autophagy machinery orchestrating the degradation of foreign invaders have skewed original notions of these pathways as exclusively "self-eating", to also effectuating xenophagy ("strange-eating")[84].

Classical macroautophagy (Figure 1.3), which we here refer to as autophagy, involves the sequestration of cytoplasmic contents in a characteristic double-membraned vacuole called the autophagosome. Fusing its outer membrane with the lysosome leads to breakdown of the inner membrane, leading to the exposure of autophagosomal content to lysosomal hydrolases which degrade them before they are released back into the cytosol for subsequent reuse[85]. Several genes have been associated with the autophagy pathway, responsible for the concertation involved in the initiation,

elongation and closure of the autophagosome, as well as its fusion with the lysosome. Beclin 1 (BECN1) for instance, has been shown to form a multimeric complex with class 3 phosphatidylinositol 3-kinase (PI3k), vacuolar protein sorting 34 (Vps34) and other proteins which is important for the nucleation of autophagosomes[86]. A series of protein classes simply termed "autophagy related" (ATG) are important for membrane localization in the generation of autophagosomes, such as ATG7 aiding two different ubiquitination (Ubi) protein conjugation systems in concertation with ATG5 and ATG12 to create ATG16 like 1 (ATG16L1), which catalyses the lipidation of ATG8 family members GABARAP/LC3 (microtubule-associated protein light chain 3) on pre-autophagosomal structures and advances their formation[87][88][89]. In humans, these are all activated downstream from the mammalian homolog of ATG1, termed uncoordinated-51-like kinases (ULK1/2), via its activation by adenosine monophosphate-activated protein kinase (AMPK). This mechanism is negatively regulated by mammalian target of rapamycin (mTOR) during nutrient-rich conditions, which subjects ULK1 to inhibitory phosphorylation modification at Ser758. Upon starvation, mTOR activity is decreased, and the site is freed for AMPK activation and subsequent ULK1/2-ATG13-focal adhesion kinase family-interacting protein of 200 kD (FIP200) complex formation, which translocates to a close domain of the endoplasmic reticulum (ER) where the aforementioned BECN1 complex may arise[90][91][92].

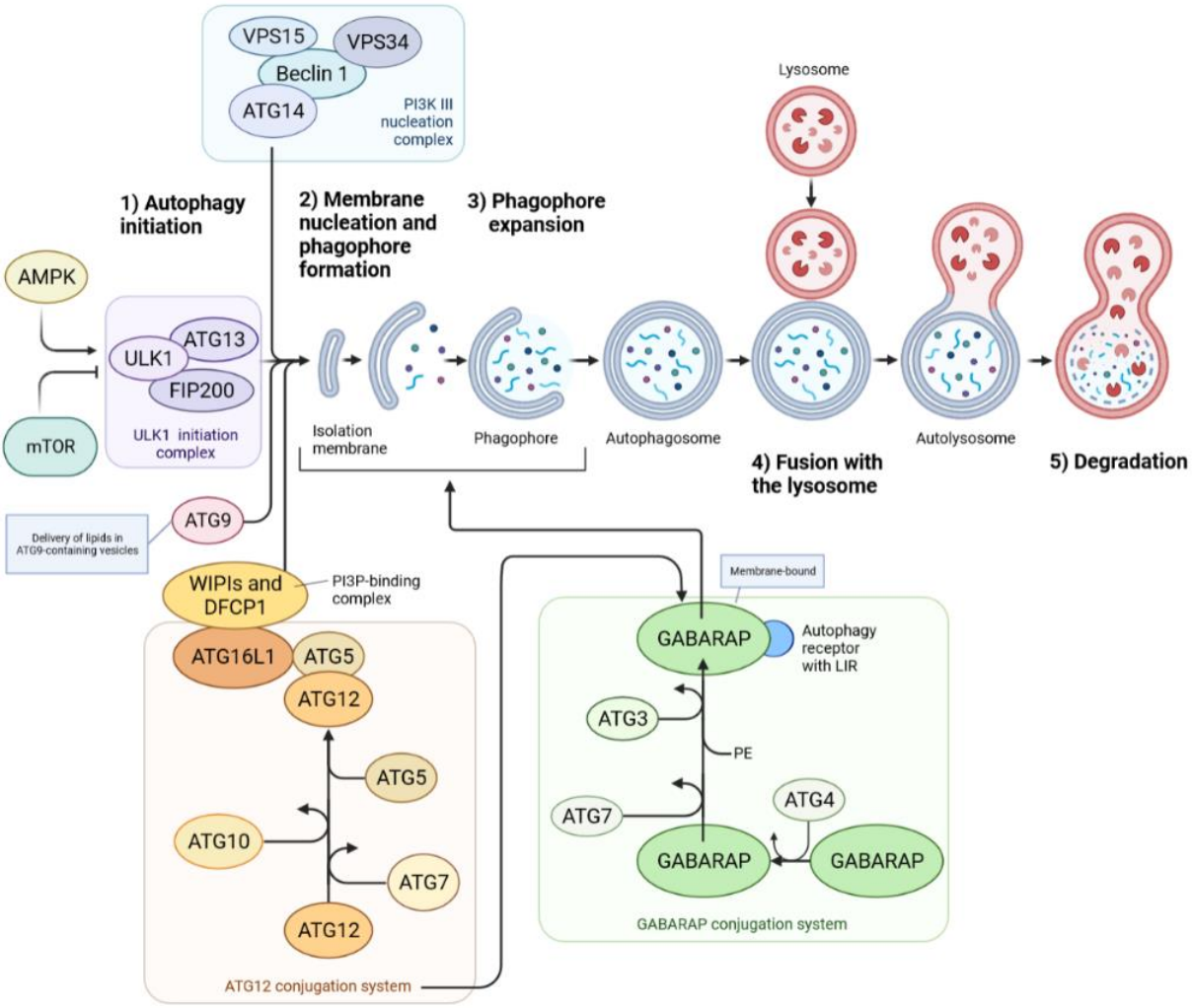


**Figure 1.3. The process of macroautophagy.** A schematic overview of the mechanical constituents forming macroautophagy, in this study referred to as autophagy, is shown. Arising with the unseating of ULK1-inhibitor mTOR, AMPK binds and activates ULK1 (or ULK2), thus recruiting its protein partners ATG13, FIP200 and ATG101 (not shown). This initiation complex is followed up by the class III PI3K nucleation complex, and the PI3P-binding complex which directs the distribution of the machinery that enables autophagosome formation. ATG12 and GABARAP/LC3 (for simplicity, only GABARAP is noted in the figure) conjugation systems are thereby recruited; ATG12 is aided by ATG7 in binding to ATG5, and forming a complex with ATG16L1, which then binds to the PI3P-binding complex and promotes the conjugation of GABARAP (or LC3), which through the stepwise aid of ATG4, ATG7 and phosphatidylethanolamine (PE) is transformed to GABARAP-II. Thereafter incorporated into pre-autophagosomal and autophagosomal membranes, GABARAP-II is associated with both their biogenesis and sealing, whilst ATG9A scrambles the phospholipids comprising their foundation[93]. Autophagosomes are fused with lysosomes for degradation. Modified from Hansen et al. using BioRender.

Besides TOR signalling, autophagy is also regulated by TLR and NLR stimulation[94]. NOD2 induces autophagy by ligating to muramyl-dipeptide (MDP), a highly conserved component of the mycobacterial cell wall which causes NOD2 to self-oligomerize, thus engaging nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways to initiate the secretion of pro-inflammatory cytokines which recruit variants in ATG16L1 to the bacterial entry site[88][95][96][97].

Having such an intertwined role in the immune system, it is of no surprise that the misregulation of autophagy is also linked to the misregulation of the immune system, such as the cases for autoimmune diseases SLE, MS and RA[98][99]. No links have yet to be made between autophagy and AAD, although the leads are many. Along with *PTPN22* which autophagy has been shown to be controlled by, the loss of *PTPN22* and the subsequent loss of autophagy regulation is linked to enhanced NF-κB and MAPK activation, leading to increased differentiation and activation of monocytes and macrophages, both which are abundant in the adrenal cortex [100][101][102]. Autophagy is also shown to play roles in the regulation of adaptive immune cells, modulating the homeostasis of T and B cells[103][104], focusing the MHC-II-peptide repertoire in TECs thereby aiding central T cell tolerance[105], and regulating the induction of co-stimulatory molecules CD80 and CD86, affecting the amount of  T cells which are allowed to escape peripheral T cell tolerance as well[106].

## Hypothesis and aims

Based on the knowledge gap indicated above the current project hypothesised that autophagy is perturbed in autoimmune Addison's disease. To elucidate this the following aims were made:

1. Compare gene expression of autophagic genes in healthy controls and AAD patients.

2. Investigate whether any changes in autophagic gene expression is related to relative abundances of neutrophils and monocytes in healthy controls and AAD patients.

3. Compare gene expression of autophagic genes in *PTPN22* high-risk and low-risk AAD patients.

4. Investigate the role of *PTPN22* in regulation of autophagy gene expression by siRNA-mediated knockdown in human monocytic cell line THP-1.

# 2. Materials

## 2.1 Chemicals and reagents

| Reagents and chemicals | Supplier | Ref.No. |
|---|---|---|
| AccuGENE 10X TBE Buffer 1L | ThermoFisher | 10358142 |
| Agarose NA | GE Healthcare | |
| Binding Buffer | PreAnalytiX | 1025506 |
| Buffer RLT | Qiagen | 1015762 |
| Buffer RW1 | Qiagen | 1014567 |
| Buffer RPE | Qiagen | 1017974 |
| CountBright™ Absolute Counting Beads | Invitrogen | C36950 |
| DEPC-Treated Water | Thermofisher | AM9915G |
| DNase I | Qiagen | 1010395 |
| DNA Digestion Buffer | Qiagen | 1010397 |
| Elution Buffer | PreAnalytiX | 1051081 |
| Ethanol Absolutt 99.9% | Killtoclean AS | 600063 |
| FACS™ Lysing Solution | BD | 15838518 |
| Fetal Bovine Serum (FBS) | Gibco | 16000-044 |
| GelRed ® 10000X in water | Biotium | 41003 |
| L-Glutamine | Sigma-Aldrich | 41003 |
| Lipofectamine™ 2000 Transfection Reagent | Invitrogen™ | 11668019 |
| Gel Loading Dye, Purple | New England Biolabs | B7024S |
| Penicillin-Streptomycin | Sigma-Aldrich | P0781 |
| Phorbol 12-myristate 13-acetate | Merck | P1585 |
| Proteinase K | PreAnalytiX | 1025497 |
| Primers | Merck | |
| RPMI Medium 1640 (1X) | Gibco | 21870-076 |
| RNase Free Water (RNFW) | PreAnalytiX | 1025498 |
| Resuspension Buffer | PreAnalytiX | 1025508 |
| UltraPure™ 10X TBE buffer | | |

| | | |
|---|---|---|
| Wash buffer 1 | PreAnalytiX | 1025505 |
| Wash buffer 2 | PreAnalytiX | 1025503 |

## 2.2 Equipment and Kits

| **Equipment and consumables** | **Supplier** | **Ref.No.** |
|---|---|---|
| 12-well Clear TC-treated plate, sterile | Costar® | 3512 |
| 384 Well PCR Plate skirted, natural | Sarstedt | 72.1984.202 |
| ART™ Barrier Reload Insert Pipette Tips: | ThermoFisher | |
| - 10 µL | | 2139-RI |
| - 100 µL | | 2065-RI |
| - 200 µL | | 2069-RI |
| - 1000 µL | | 2179-RI |
| Cell Scraper | Corning Inc. | 3010 |
| Collection Tube (1.5 mL) | Qiagen | 1017981 |
| Collection Tube (2.0 mL) | Qiagen | 1016810 |
| Falcon tubes: | Corning Inc. | |
| - 15 mL | | 525-0150 |
| - 50 mL | | 525-0156 |
| Finnpipette F1 pipettes: | ThermoFisher | |
| - 0.2 – 2 µL | | 4641020N |
| - 0.5 – 5 µL | | 4641010N |
| - 1 – 10 µL | | 4641030N |
| - 2 – 20 µL | | 4641050N |
| - 10 – 100 µL | | 4641070N |
| - 20 – 200 µL | | 4641080N |
| - 100 – 1000 µL | | 4641100N |
| Cell Sorting tube (5.0 µL) | Sarstedt | 55.1578 |
| High-Capacity RNA-to-cDNA™ Kit | ThermoFisher | 4387406 |
| MicroAmp™ Optical Adhesive | ThermoFisher | 4311971 |

| | | |
|---|---|---|
| Film | | |
| Microcentrifuge Tube (1.5 mL) | PreAnalytiX | 1025487 |
| Nitrile Medical Examination Gloves M | Abena | 290498 |
| PAXgene Blood RNA Kit | PreAnalytiX | 762174 |
| PAXgene Blood RNA Tubes | PreAnalytiX | 762165 |
| PAXgene RNA spin column | PreAnalytiX | 1028922 |
| PAXgene Shredder spin column | PreAnalytiX | 1028923 |
| PowerTrack™ SYBR Green Master Mix | Thermofisher | A46109 |
| Processing Tube (2 mL) | PreAnalytiX | 1025488 |
| QIAshredder Mini Spin Column | Qiagen | 1011711 |
| RNeasy Mini kit | Qiagen | 74104 |
| RNeasy Mini spin column | Qiagen | 1112543 |
| | | |
| Secondary BD Hemogard Closure | Qiagen | 1028920 |
| THP-1 Cells | ATCC | TIB-202™ |
| The TriFECTa® Kit | IDT | |
| Vacutainer™ 2 mL. Heparin Tubes | BD | 121557 |

## 2.3 Instruments

| **Instrument** | **Supplier** | **Ref.No.** |
|---|---|---|
| CKX53 Cell Culture Microscope | Olympus | N8600216-102015 |
| EPS 301 Electrophoresis Power Supply | Cytiva | 10076004 |
| Gel Doc™ EZ Imager | Bio-Rad | 1708270 |
| HE 33 Mini Submarine Electrophoresis Unit | Hoefer™ | HE33-8-1.5 |
| Heraeus Fresco 21 Centrifuge | ThermoFisher | 41056779 |
| Heraeus Multifuge 3SR+ | ThermoFisher | 40787159 |
| Mars Safety Class 2 Cabinet | Scanlaf | 9.002.022.000 |

| | | |
|---|---|---|
| ND-1000 Spectrophotomer | NanoDrop | Saveen Werner |
| Sanyo MCO-19AIC CO2 Incubator | Sanyo | LV42443983 |
| Thermomixer | Eppendorff | 5350 |
| Unimax 1010 Shaker Incubator | Heidolph | 543-12310-00 |
| Vortex mixer | VWR | VVRI444-1372 |
| Veriti™ Dx 96-well Thermal Cycler, 0.2 mL | Applied Biosystems™ | 4452300 |
| QuantStudio™ 5 Real-Time PCR System, 384-well | Applied Biosystems™ | A28140 |

**Software**

| **Program** | **Supplier** |
|---|---|
| BioRender | BioRender |
| GraphPad Prism 9.3 | |
| Image Lab 6.01 | Bio-Rad |
| Primer-Blast | NCBI |
| Microsoft Excel 2016 | Microsoft |
| Microsoft Paint | Microsoft |
| ND-1000 v3.8.1 | Saveen Werner |
| FlowJo 10.8.1 | LLC |
| QuantStudio™ Design & Analysis Software | ThermoFisher |

# 3. Methods

## 3.1 Experimental pipeline

To investigate the aims described above, blood from AAD patients and healthy donors was used to study gene expression and abundance of certain blood cell populations, while a cell line was used to test causality of PTPN22 in regulation of autophagy genes (Figure 3.1).

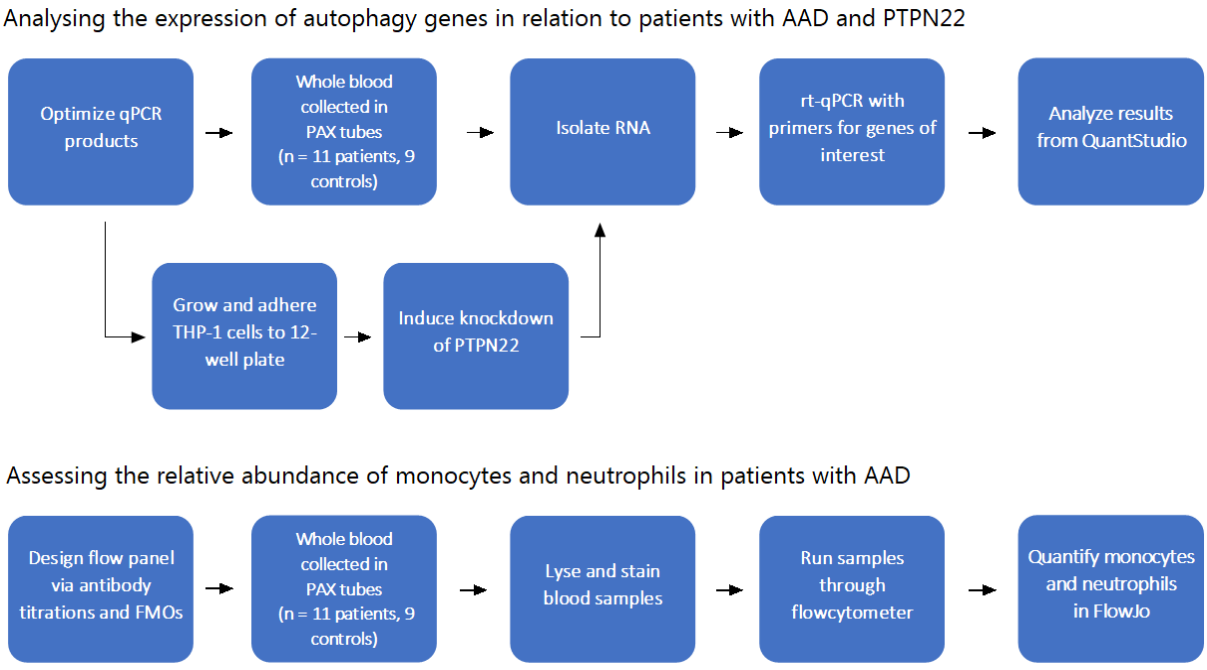


**Figure 3.1: Overview of the experimental pipeline.**

## 3.2 Methodological Considerations

The main techniques used in this project are real-time PCR (qPCR) and flow cytometry. qPCR is well-established as a method of detecting and quantifying nucleic acid molecules, such as the those of our genes of interest. Reverse transcription PCR of isolated RNA generates complementary double-stranded (ds)DNA which can be bound by the fluorescent dye SYBR Green, detectable by a real-time PCR system. Indeed, as more double-stranded amplicons are produced in the event of a PCR, SYBR Green fluorescence increases correlatively [107]. In real-time PCR, this is marked by using a cycle

threshold ($C_t$), which is the number of PCR cycles necessary to produce a fluorescent signal exceeding the background level. As $C_t$ values of each gene are directly corresponding to their amounts in solution, one can thereby use $\Delta\Delta C_t$ -method (further described in 3.9) to assess their fold change and relative expression [108].

Flow cytometry allows for rapid multi-parametric analysis of cells in solution. Its use of lasers as light sources produces both scattered and fluorescent signals, allowing for the detection of size, complexity, and multiple fluorescence parameters in single cells at the same time. By creating a panel of relevant antibodies to detect neutrophils and monocytes, flow cytometry is rendered an efficient way of detecting their relative abundance in samples of whole blood by gating for these parameters [109].

The results gained from qPCR data of donors was further tested for causality in vitro, by using the human monocytic cell line THP-1. Since the test involved gene knockdown by siRNA transfection, THP-1 was deemed a suitable choice of cell line as the monocytes can be adhered to cell culture plates, making the transfection process with Lipofectamine™ 2000 more convenient.

## 3.3 Patients and Controls

For determining the gene expression of chosen autophagy genes, whole blood from 10 patients (all females, age range 59-78, mean age 65.9) and 11 healthy controls (9 females, 2 males, age range 51-62, mean age 55.5) were used. Determining the relative abundances of monocytes and neutrophils in patients with AAD compared to healthy controls was carried out with whole blood from 14 patients (6 females, 8 males, age range 17-68, mean age 50) and 12 healthy controls (6 females, 6 males, age range 21 – 63, mean age 50). Of all samples, only the patients used in the autophagy gene expression experiments had known alleles for *PTPN22*; these are listed in Table A.1, along with the sex and age of each individual donor.

## 3.4 Ethical Aspects

The study was approved by the Regional Ethical Committee of Western Norway with the approval number 2018/1417. Samples were recruited from the Registry and biobank for organ specific autoimmune disorders (ROAS), Haukeland University Hospital, Norway, with the approval number 2013/1504); all donors gave written informed consent for participation. Control samples were obtained from the Haukeland University Hospital blood bank and had signed consent for research.

## 3.5 THP-1 differentiation and PTPN22 knockdown

Cells from the human monocyte leukaemia cell line THP-1 (ATCC), cultured with growth medium RPMI 1640 (10% FBS (Gibco), 2% L-Glutamine (Sigma-Aldrich), 1% Penicillin-streptomycin (Sibma-Alrdrich)), were made adherent as described by Caras et al [110]. In short, this was achieved by treating the cells for 24h with 30 ng/mL phorbol 12-myristate 13-acetate (PMA) (Merck) in 12-well cell culture plates (Costar ®) with 1 mL cell suspension (0.5 x $10^6$ cells) in each well. After the 24h, adherent cells were washed once with growth medium then again added 2 mL growth medium.

*PTPN22* knockdown (KD) was carried out using the TriFECTa® Kit (IDT), with Dicer-Substrate siRNA predesigned for *PTPN22*, as well as control siRNA and buffer. Transfection of siRNA was carried out with Lipofectamine™ 2000 Transfection Reagent. siRNA and Lipofectamine™ were diluted separately in serum free RPMI 1640: 5 nM siRNA for solution A, and 0.02 µL Lipofectamine™ per µL RPMI 1640 for solution B. Each solution was mixed gently and incubated for 5 minutes in room temperature (RT) before being combined and further incubated for 20 minutes in RT. After 20 minutes, the 12-well cell culture plates had their media removed and replaced with 1.5 mL serum free RPMI 1640 and 500 µL combined solutions A+B. The plates were mixed gently by rocking the plates and incubated in incubator (37°C with 5% $CO_2$) for 4 hours. Transfection medium was then replaced with growth medium (10% or 1% FBS), and the plates further incubated in incubator for 24 hours.

## 3.6 RNA isolation from THP-1 cells

RNA was isolated from the THP-1 cells using an RNeasy Mini kit from Qiagen according to their protocol. Briefly, the cell culture medium was removed, and the monolayer disrupted using 600 µL Buffer RLT lysis buffer and a cell scraper (Corning). The lysate was transferred to a QIAshredder spin column and centrifuged for 2 min at full speed. The homogenized lysate was added 1 volume of 70% ethanol and mixed by pipetting, before being transferred to an RNeasy spin column and centrifuged for 15 s at 8000 x *g*. The flow-through was discarded, the spin column added 700 µL Buffer RW1, then centrifuged for 15 s at 8000 x *g*. The flow-through was discarded, the spin column added 500 µL Buffer RPE, then centrifuged for 15 s at 8000 x *g* before repeating this step. Finally, the RNA was eluted from the spin column into a fresh 1.5 mL collection tube by adding diethyl pyrocarbonate-treated RNase free water (RNFW) directly onto the spin column membrane and centrifuging for 1 min at 8000 x *g*. Eluted RNA was stored at – 80°C.

## 3.7 RNA isolation from whole blood

Blood from eleven patients with Addison's disease as well as nine healthy controls was drawn in individual PAXgene Blood RNA Tubes (BRTs) (Qiagen) and had RNA isolated via the PAXgene Blood RNA Kit (Qiagen) following the manufacturer's protocol. Briefly, BRTs were incubated for 2 hours at RT after blood collection, then centrifuged at 3000 x *g* for 10 minutes. The supernatant was removed, and the pellet added 4 mL RNFW. The tube was closed with a fresh Secondary BD Hemogard Closure and vortexed until the pellet was dissolved, then centrifuged for 10 minutes at 3000 x *g*. New supernatant was removed and 350 µL resuspension buffer was added, and the tube vortexed until the pellet was dissolved. The sample was then pipetted to a 1.5 mL microcentrifuge tube, added 300 µL binding buffer and 40 µL proteinase K, and mixed by vortexing for 5 seconds before being incubated for 10 minutes at 55°C using a shaker-incubator (Heidolph) at 800 rpm. The lysate was then pipetted into PAXgene Shredder spin column placed in a 2 mL processing tube (PT), and centrifuged for 3 minutes at 20 000 x *g*. The supernatant was transferred to a fresh microcentrifuge tube, added 350 mL ethanol (96%), mixed by vortexing and centrifuged for 2 seconds at 800 x *g*, and pipetted into a PAXgene RNA spin column (PRC) placed in a PT. The PRC was then centrifuged for 1 minute at 20 000 x *g*. The PT was replaced with a fresh one, and the PRC added 350 µL wash buffer 1 before being centrifuged for 1 minute at 20 000 x *g* and placed into a fresh PT. An incubation mix created by mixing 10 µL DNase I and 70 µL DNA digestion buffer was pipetted directly onto PRC membrane and left to incubate on the benchtop (20-30°C) for 15 minutes. In the subsequent steps the PRC was sequentially added 350 µL wash buffer 1, 500 µL wash buffer 2, and 500 µL wash buffer 2 again, centrifuging at 20 000 x *g* for 1, 1 and 3 minutes respectively, replacing the PT with a fresh one after each step. RNA was then eluted into a microcentrifuge tube which the PRC was placed into, by pipetting 40 µL elution buffer and centrifuging for 1 minute at 20 000 x *g*, a step which was repeated to elute remaining RNA from the PRC. The microcentrifuge tube was then incubated for 5 minutes at 65°C before being immediately chilled and stored at – 80°C.

## 3.8 Quality checking of qPCR product with agarose gel electrophoresis

To carry out gel electrophoresis of qPCR products, 1% agarose (GE Healthcare) was mixed with 1X Tris-borate-EDTA (TBE) (ThermoFisher). The solution was microwaved until visibly dissolved and then allowed to cool down for 3 minutes before being added 1X GelRed Nucleic Acid Stain (Biotium). The ready solution was poured into a gel tray with the well comb in place and allowed to set for 30 minutes. After solidification the agarose gel was placed into the electrophoresis unit (Hoefer$^{TM}$) and filled up with 1X TBE until it was completely submerged.

Three µL Generuler 1 kb Plus DNA ladder was added to the first and last well, while the remaining wells were loaded with 10 µL qPCR product mixed with 1 µL gel loading dye (New England Biolabs. The gel was run at 200V, 220 mA for 30 minutes, before being moved to the Gel Doc™ EZ Imager for image taking and analysing via Image Lab (Bio-Rad).

## 3.9 Profiling gene expression related to autophagy

The concentrations of RNA isolated were measured using NanoDrop 1000 Spectrophotometer (ThermoFisher) (Table A.2), allowing for equal amounts of RNA (0.5 µg from all patient and control samples, 1.0 µg from all THP-1 samples) to be used for complementary DNA (cDNA) synthesis using a High-Capacity RNA-to-cDNA™ Kit (ThermoFisher). This was done according to the manufacturer's protocol with the aid of a Veriti 96 well Thermal Cycler (ThermoFisher). Primers for 11 autophagy genes were chosen, as well as the housekeeping gene GAPDH (Table 3.1). They were all designed in Primer-Blast and ordered from Merck. Primer mixes were thus prepared with 2x PowerTrack™ SYBR Green Master Mix (ThermoFisher) and 5 µM forward (fwd.) + reverse (rev.) primer.

**Table 3.1: Primers used in the study.**

| Primer | Fwd. sequence | Rev. sequence | Size (bp) |
|---|---|---|---|
| ULK1 | GTTCCAAACACCTCGGTCCT | CCAACTTGAGGAGATGGCGT | 168 |
| BECN1 | GAAGAGCATCGGGGGCTGAG | GTGGTAAGTAATGGAGCTGTGAGT | 168 |
| ATG5 | GGAAGCAGAACCATACTATTTGC | AATGCCATTTCAGTGGTGTGC | 152 |
| ATG7 | CGGGATTTCTAGAGCAGCCT | ATGGGGTTTGAGAAGCCTTGAT | 94 |
| GAPDH | AGGTCGGAGTCAACGGATTT | TGGAATTTGCCATGGGTGGA | 157 |
| ULK2 | CACCTTCCCCTTTGGTTGGT | GAGTCATGGCCCTGAGGATG | 76 |
| ATG9A | CATACTGTCCATGGAGCTGGTGG | GGCCTCTAGGCGCTGGTATT | 87 |
| ATG12 | GTGGCCTCAGAACAGTTGTTTA | CACGCCTGAGACTTGCAGTA | 134 |
| GABARAP | AAGAAGAGCATCCGTTCGAGA | TTCTACTATCACCGGCACCC | 86 |
| PIK3C3 | CCGCTGTAGGTGGTACCTTT | CTTCCCTTCCAAGCTTCCTATCT | 119 |
| MAP1LC3B | AGCATCCAACCAAAATCCCG | AGCTGTAAGCGCCTTCTAAT | 142 |
| ATG4B | CTGCGGTGTGGACAGATGAT | GTGTCCACCTCCAATCTCGG | 73 |

Primer-master mix solutions were added to cDNA samples (1.5 ng/µL) as well as non-template controls (NTCs) for total volumes of 10 µL in triplicates in 384-well plates (Sarstedt). Loaded plates were run for 40 cycles on a QuantStudio™ 5 Real-Time PCR System (Table 3.2).

**Table 3.2: Program used to run qPCR on the QuantStudio™ 5 Real-Time PCR System.**

| Stage | Temperature | Time | Cycles |
|---|---|---|---|
| 1 | 50°C | 2 minutes | 1 |
| 2 | 95°C | 10 minutes | 40 |
| 3 | 95°C | 15 seconds | |
| | 60°C | 1 minute | |

The $C_t$s provided by QuantStudio™ Design and Analysis Software were transferred to Excel (Microsoft) for fold change calculation using the $\Delta\Delta C_t$ -method[111]. This involved normalizing the triplicates of $C_t$s in reference to the housekeeping gene in order to gain a $\Delta C_t$ value. The data was then further normalized by subtracting $\Delta C_t$ values with the mean $\Delta C_t$ value of the control group, gaining $\Delta\Delta C_t$. Finally, the fold change was calculated as $2^{-\Delta\Delta Ct}$, which represents the relative change in expression for the gene being analysed. An example for the calculation of fold change is displayed in Table 3.3. A value below 1 means that the gene is downregulated, while a value above 1 means the gene is upregulated [111].

**Table 3.3. An example of fold change (FC) calculation for ATG5.**

| Sample | Allele | GAPDH | ATG5 | ΔCt | Mean | ΔΔCt | FC | FC average | Ttest |
|---|---|---|---|---|---|---|---|---|---|
| P4 | A2/A2 | 22.46 | 21.49 | -0.97 | -2.25 | 1.28 | 0.41 | | |
| P8 | A2/A2 | 21.99 | 19.85 | -2.14 | | 0.11 | 0.93 | | |
| P9 | A2/A2 | 22.53 | 19.57 | -2.97 | | -0.72 | 1.65 | | |
| P10 | A2/A2 | 22.02 | 19.19 | -2.83 | | -0.59 | 1.50 | | |
| P11 | A2/A2 | 24.30 | 21.98 | -2.32 | | -0.08 | 1.05 | 1.11 | |
| P1 | A1/A1 | 24.56 | 21.09 | -3.47 | - | -1.22 | 2.33 | | |
| P2 | A1/A1 | 23.24 | 19.21 | -4.03 | | -1.78 | 3.44 | | |
| P3 | A1/A1 | 23.78 | 20.43 | -3.35 | | -1.11 | 2.15 | | |
| P5 | A1/A2 | 24.48 | 20.53 | -3.95 | | -1.71 | 3.26 | | |
| P6 | A1/A2 | 25.13 | 21.47 | -3.67 | | -1.42 | 2.67 | | |
| P7 | A1/A2 | 22.72 | 18.45 | -4.27 | | -2.02 | 4.05 | 3.04 | **0.00085** |

## 3.10 Determining ratios of neutrophils and monocytes in whole blood

The relative abundance of neutrophils and classical, non-classical and intermediate monocytes were detected in patients with AAD and healthy controls using flow cytometry. Whole blood was collected in heparin tubes (BD) and 100 µL transferred to fluorescence activated cell sorting (FACS) tubes. Further sample preparation involved staining with antibodies and vortexing before incubation for 15 minutes at RT in the dark, then adding 900 µL lysing buffer (BD) and vortexing before further 15 minutes of incubation at RT in the dark. The final sample volume was 1000 µL.

Optimization of flow data involved several steps, thereof titration of the antibodies. Deciding the appropriate concentrations to use is a vital step in ensuring the best resolution, as too little antibody leaves the specific positive signal to be weak, while too much antibody causes an increase in the amount of non-specific binding, as well as the spread and background of negative populations [112] [113]. This was carried out by single staining solutions CD88 (1:4000, 1:2000, 1:1000 and 1:500), CD45 (1:400, 1:200, 1:100 and 1:50) and HLA-DR (1:5000, 1:2500, 1:1250 and 1:625). CD89, CD14 and CD16 had already been titrated by the research group. The values determined to use in the panel are provided in Table 3.4.

**Table 3.4: Antibodies chosen for staining of full blood.** In order to determine the suitable dilution whole blood, titration was carried out.

| Antibody | Fluorochrome | Clone | Stock dilution | Dilution chosen for 100 µL whole blood | Biolegend catalogue ID |
|---|---|---|---|---|---|
| CD45 | BC785 | HI30 | 1:2 | 1:100 | 368527 |
| HLA-DR | FITC | L243 | 1:20 | 1:1000 | 307604 |
| CD88 | APC Fire 750 | S5/1 | 1:16 | 1:800 | 344316 |
| CD89 | APC | A59 | 1:4 | 1:200 | 354106 |
| CD14 | PE | M5E2 | 1:20 | 1:1000 | 301806 |
| CD16 | BV605 | 3G8 | 1:2 | 1:100 | 302040 |

The other steps for optimizing the panel were using fluorescence minus one (FMO) controls for more accurate gating and compensating spectral overlap via single staining. All FACS samples were run on a LSRFortessa™ flow cytometer (BD) and analysed in FlowJo (LLC), from which events of relevant gates were extracted to Excel for the calculation of relevant ratios.

## 3.11 Statistics

For statistical analysis of qPCR and flow cytometry data, unpaired T tests were used, where independent groups are compared in a parametric test with the assumption that the values follow a Gaussian distribution. For data derived from blood of patients and healthy controls, comparisons were made without the assumption that the samples had the same standard deviation, and Welch's T test was applied. This follows the natural variation of gene expression between individual persons. On the other hand, data from the clonal THP-1 cell line were assumed to have the same standard deviations, and therefore not applied Welch's T test. A significant level of $P < 0.05$ was considered statistically significant. All statistical analysis was carried out by using GraphPad Prism 9.3.

# 4. Results

## 4.1 Analysing autophagy gene expression

Eleven genes involved in the macroautophagy pathway, namely *ULK1*, *ULK2*, *ATG9A*, *ATG12*, *GABARAP*, *PIK3C3*, *ATG4B*, *ATG5*, *ATG7*, *BECN1* and *MAP1LC3B*, as well as the housekeeping gene *GAPDH*, were selected in order to study the role of autophagy in patients with AAD. This was carried out by using whole blood from donors with AAD and healthy controls (Table A.1).

### 4.1.1 Optimization and quality control of qPCR products

Prior to analysing the gene expression of AAD patients and controls via qPCR, the suitable amount of cDNA was defined for the qPCR assay. Primers for each of the 11 chosen autophagy genes as well as *GAPDH* were tested with cDNA amounts of 3, 6, 9 and 12 ng, following the given recommendation of 1-10 ng cDNA in the PowerTrack™ SYBR™ Green Master Mix user guide. Resulting qPCR data was provided by QuantStudio™ Design & Analysis Software and extracted to Excel, where line charts for each primer were created based on ng cDNA added and the $C_t$ for the given value (Figure 4.1). The $C_t$s ranged between 28 for the lowest amount of *BECLIN1* and 18 for the highest value of *ATG12*. In fact, all of the added amounts of cDNA deemed $C_t$s within the linear dynamic range for qPCR (15-30) [114][115]. Following this, 10 ng cDNA was chosen as a reliable amount to be used in the following experiments for all the chosen genes.

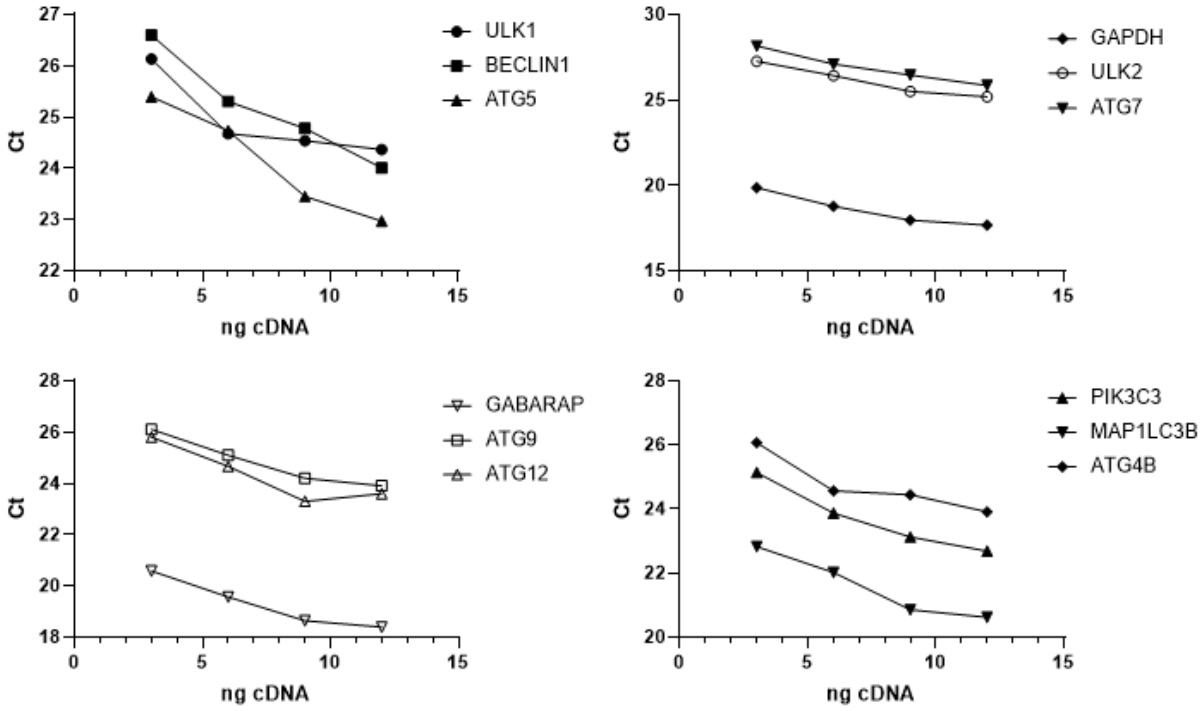


**Figure 4.1 Defining an optimal amount of cDNA to be used in real time qPCR with the chosen genes.** 3, 6, 9 and 12 ng cDNA were used according to the PowerTrack™ SYBR™ Green Master Mix user guide. The linear dynamic range of qPCR is defined to be between 15-30 $C_t$.

The qPCR products were further controlled via gel electrophoresis in order to examine the specificity of the primers to the genes. Strong bands were exhibited for all samples shown in Figure 4.2, and their sizes based on migration matched the sizes of the expected PCR product (Table 3.1). All PCR reactions showing one strong band in the PCR product indicates specificity for the primers. All samples also showed an extra set of weaker bands further below in the gel, a result of excess primers which weren't used during qPCR.

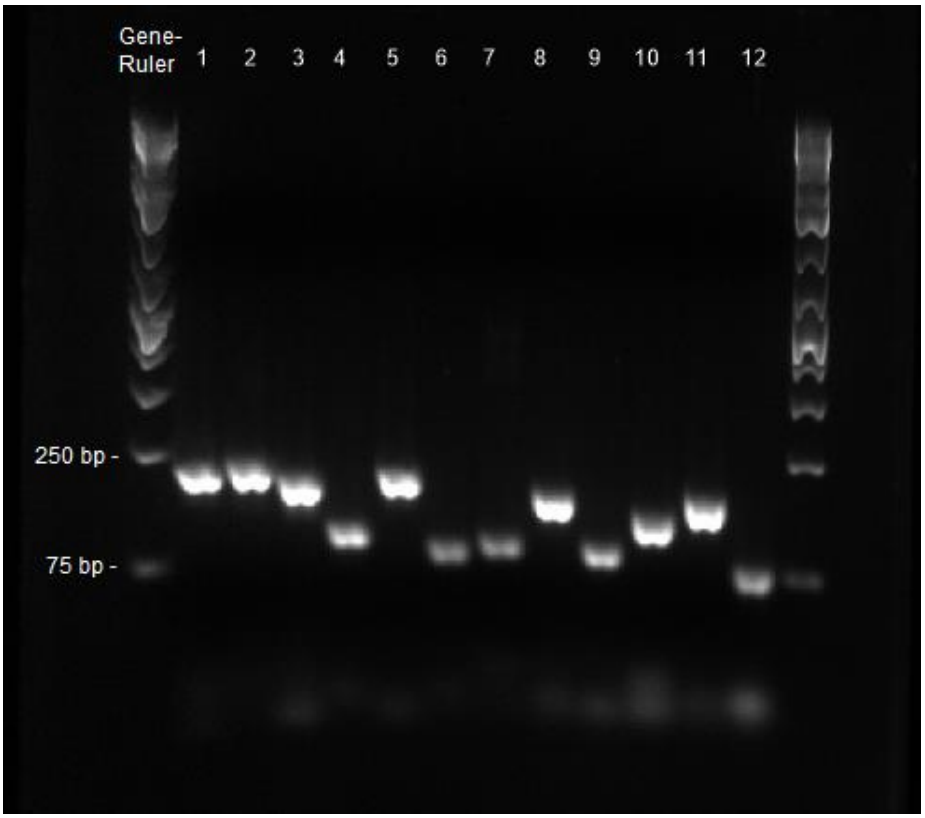


**Figure 4.2. Agarose gel showing the specificity of the chosen primers.** Listed on top of the figure, 1-12 represent qPCR products of *ULK1*, *BECN1*, *ATG5*, *ATG7*, *GAPDH*, *ULK2*, *ATG9A*, *ATG12*, *GABARAP*, *PIK3C3*, *MAP1LC3B* and *ATG4B*, respectively. The image was taken using a GelDoc™ EZ Imager (Bio-Rad) and processed in ImageLab (Bio-Rad).

### 4.1.2 ULK1, ULK2, ATG9A, ATG12 and GABARAP show different expression levels in whole blood of AAD patients compared to controls

The expression levels of the 11 autophagy genes were then analysed in AAD patients and controls via real-time qPCR. Figure 4.3 shows the fold change of chosen autophagy genes in whole blood of patient and controls, and their variance. *ULK1*, *ULK2*, *ATG9A* and *ATG12* were expressed at significantly higher levels in patients with AAD compared to controls, whilst GABARAP was expressed at a significantly lower level. In contrast, *PIK3C3*, *ATG4B*, *ATG5*, *ATG7*, *BECN1* and *MAP1LC3B* showed no significant change in gene expression between the groups.

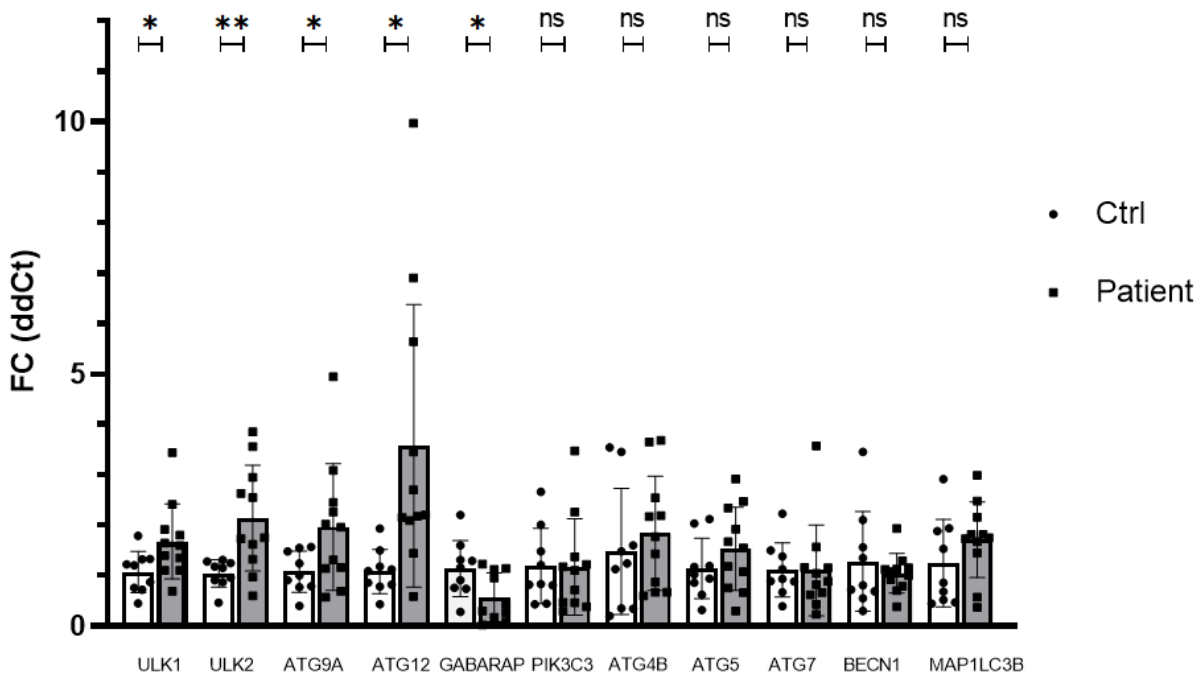


**Figure 4.3: Autophagy gene expression in AAD-patients and control.** Relative to control (white columns), AAD patients (grey columns) were found to have higher levels of *ULK1*, *ULK2*, *ATG9A* and *ATG12*, and lower levels of *GABARAP*. A total of 9 patients and 11 controls were used for each gene in triplicates for each reaction. The $C_t$s assessed via real-time qPCR and presented by QuantStudio™ Design & Analysis Software were extracted to Excel, where fold change (FC) was calculated by using the $2^{-\Delta\Delta Ct}$-method with *GAPDH* as the reference gene. * = $p \leq 0.05$, ** = $p \leq 0.01$, ns = not significant, unpaired T-test with Welch's correction for unequal variance.

## 4.2 Assessing the ratios of neutrophils and monocytes in AAD-patients and control with flow cytometry

Since some of the autophagy genes found upregulated AAD patients are known to be highly expressed in neutrophils and monocytes, such as *ULK1*, *ULK2*, and *ATG9A* [116][117], flow cytometry was used to assess whether these changes in expression were associated with a perturbed abundance of neutrophils and/or monocytes in blood. To achieve this, a flow cytometry panel was developed as described in the following sections.

### 4.2.1 Determination of antibody concentrations

The various antibodies chosen to determine neutrophil and monocytes quantities were titrated in order to determine their optimal concentrations. Resulting data obtained from the cell suspensions by the flow cytometer were analysed in FlowJo, where a large separation in positive and negative populations was prioritized. Figure 4.4 shows gating and histograms for CD88, CD45 and HLA-DR, while Figure

4.5 show their resulting titration and the ratios obtained upon comparison of positive and negative populations.

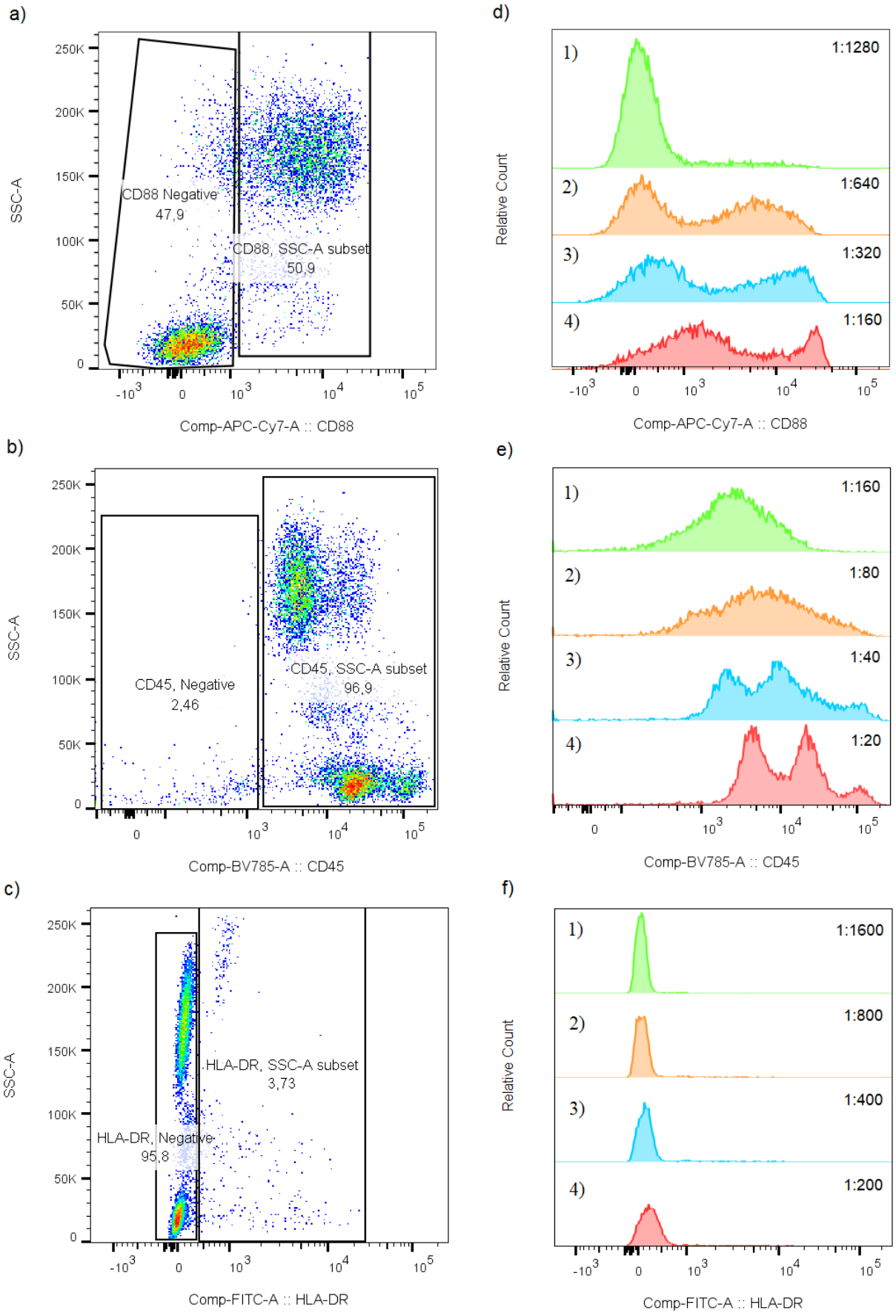


**Figure 4.4: Gating and histograms for CD88, CD45 and HLA-DR.** Different concentrations of the antibodies were single stained in solutions of whole blood and analysed in FlowJo. **A), b) and c)** show cytograms of CD88 (1:320), CD45 (1:160)

and HLA-DR (1:200), respectively, where the y-axis shows the side scatter (SSC-A), and the x-axis shows the signal intensity. The gates to the right define positive populations of said antibody whilst the gates to the left define negative populations. **D), e) and f)** show histograms of antibody titration for CD88, CD45 and HLA-DR, respectively, where the y-axis shows the relative count of cells, and the x-axis shows the signal intensity. Samples 1-4 contain in **d)** antibody concentrations of 1:1280, 1:640, 1:320 and 1:160, respectively, in **e)** 1:160, 1:80, 1:40 and 1:20, respectively, and in **f)** 1:1600, 1:800, 1:400 and 1:200, respectively.

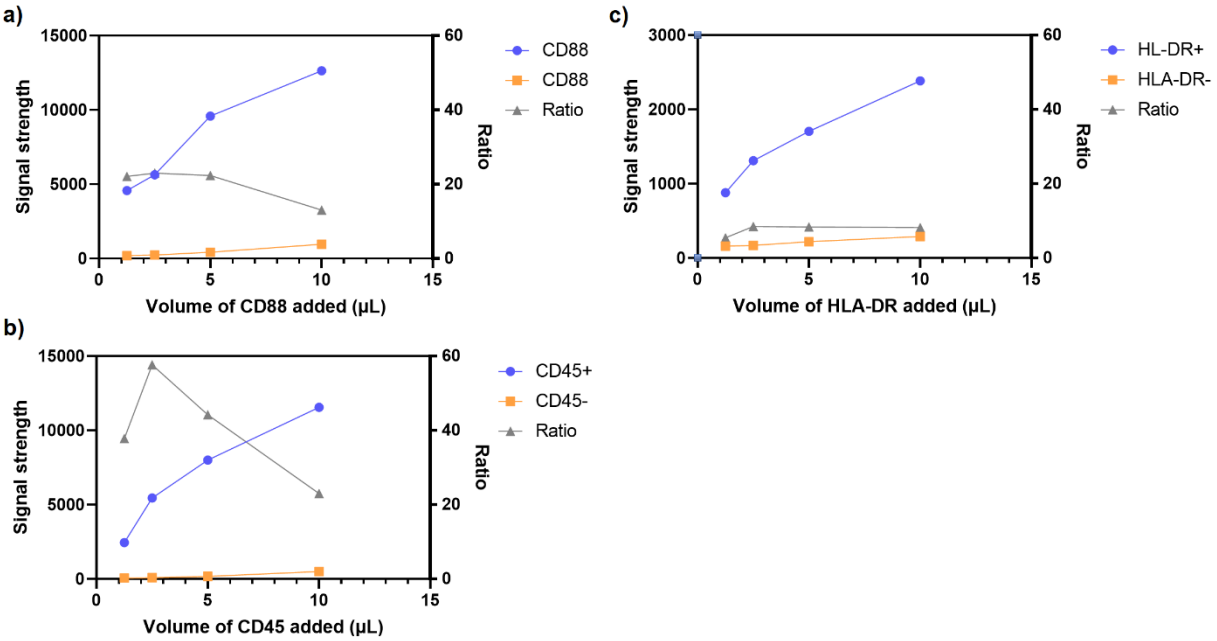


**Figure 4.5: Titration of antibodies.** CD88 (stock solution 1:16), HLA-DR (stock solution 1:20) and CD45 (stock solution 1:2) were added in volumes of 1.25, 2.5, 5 and 10 µL to individual solutions of 100 µL whole blood before having their signals measured by flow cytometry. Positive (+) and negative (-) populations were gated in the manner of Figure 4.5a), and the resulting values plotted in line charts along with the ratios between them. **A), b)** and **c)** show the results for CD88, CD14 and HLA-DR, respectively, with + and – populations plotted on the left y-axis, while the ratios between them were plotted on the right y-axis. Of the chosen volumes (shown in the x-axis), staining with 2.5 µL antibody deemed the highest ratio for + to – populations in all three antibodies.

In Figure 4.5, the highest ratios were obtained with 2.5 µL CD88, CD45 and HLA-DR, equivalent to 1:640, 1:80 and 1:800 in 100 µL whole blood, respectively. Although the manufacturer of the antibodies recommended 5 µL in 100 µL whole blood, lower concentrations were found to be optimal for all antibodies. For simplicity, 2 µL was chosen to be used in the panel. The final dilutions of the antibodies used in the flow cytometry panel are shown in Table 3.4.

### 4.2.2 Gating strategy

Before analysing patient and control samples, gating strategies were developed to identify the populations of interest. Neutrophils were defined as CD16+; CD88+, and the different monocyte subsets were defined as CD14+; CD16-, CD14+; CD16+ and CD14-; CD16+ for classical, intermediate and non-classical monocytes, respectively. Figure 4.6 shows the strategy for identifying

neutrophils, and Figure 4.7 shows the strategy for identifying the subsets of monocytes. FMOs were utilized to identify positive populations. All samples followed the same strategies.

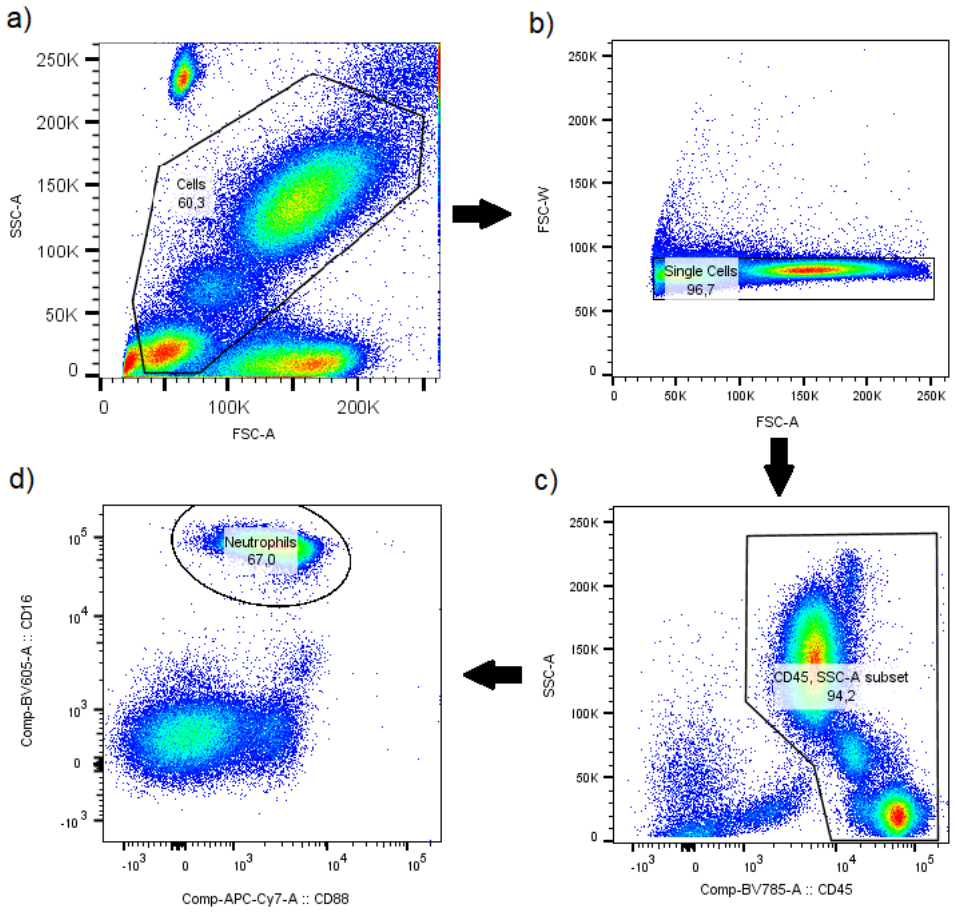


**Figure 4.6: Gating strategy for neutrophils. a)** Events captured by the BD LSRFortessa$^{TM}$ flow cytometer are displayed in FlowJo as plot of forward scatter area (FSC-A) versus side scatter area (SSC-A). The polygon gate marks "Cells", continuing gating to **b)** where FSC-A versus FSC width (FSC-W) allows for the rectangular gate of single cells. Further gating of $CD45^{+}$ are carried out in **c)** with a plot of BV785 fluorescence versus SSC-A, before the gating of neutrophils in **d)** with APC Fire 750 versus BC605.

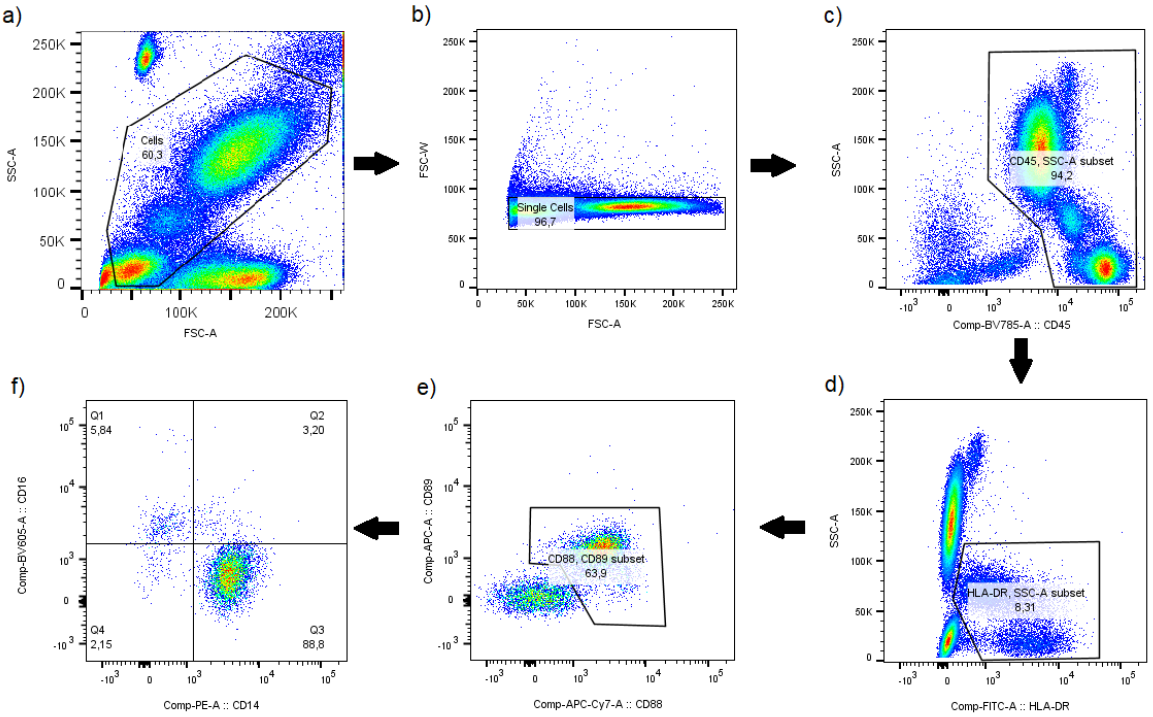


**Figure 4.7: Gating strategy for neutrophils. a)** Events captured by the BD LSRFortessa$^{TM}$ flow cytometer are displayed in FlowJo as plot of forward scatter area (FSC-A) versus side scatter area (SSC-A). The polygon gate marks "Cells", continuing gating to **b)** where FSC-A versus FSC width (FSC-W) allows for the rectangular gate of single cells. Further gating of CD45$^{+}$ is carried out in **c)** with a plot of BV785 fluorescence versus SSC-A, before the gating of HLA-DR$^{+}$ in **d)** with FITC versus SSC-A. CD88$^{+}$; CD89$^{+}$ are gated in **e)** with APC Fire 750 versus APC, the subsets of monocytes are gated in **f)** with PE vs BV605. Q1, Q2, and Q3 represent non-classical, intermediate and classical monocytes, respectively.

### 4.2.3 Relative abundance of neutrophils and monocytes in AAD patients and healthy controls

Using the mentioned panel, the ratios of neutrophils and monocyte subsets were calculated relative to leukocytes, set as CD45+ (marked in Figure 4.6- and 4.7c), in AAD patients and control. Figure 4.8 shows the ratios of classical, intermediate and non-classical monocytes, as well as neutrophils, in patients with AAD and controls. No significant change was detected between the groups, suggesting that the differences in autophagy gene expression does not stem from an altered abundance of monocytes or neutrophils in this patient group.

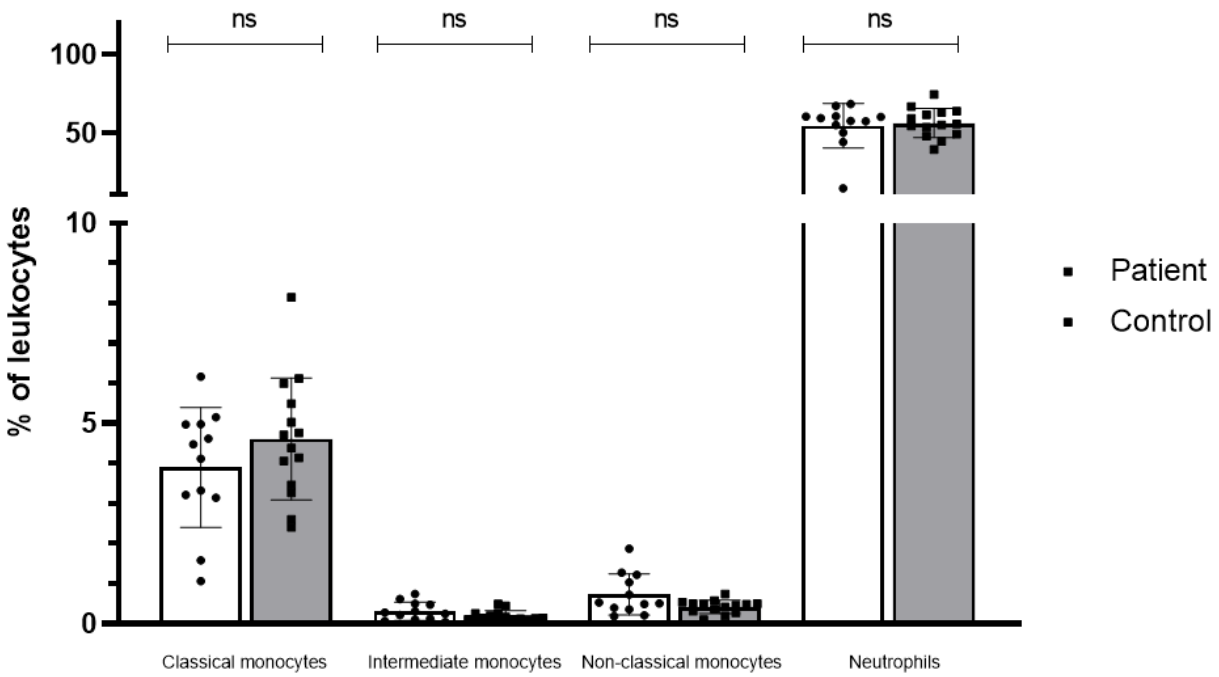


**Figure 4.8: Patients with AAD have no significant change in ratios of monocytes and neutrophils to leukocytes.** Comparison between monocyte and neutrophil ratios to leukocytes in patients and control was analysed using Welch's T-test. The results showed no significant changes in the ratios of classical, intermediate and non-classical monocytes, neither in neutrophils. ns = not significant, unpaired T-test with Welch's correction for unequal variance.

## 4.3 PTPN22 risk allele is associated with ATG5, ATG12 and GABARAP in whole blood of AAD-patients

To assess whether the *PTPN22* risk allele is associated with autophagy gene expression in whole blood of patients with AAD, another fold change comparison was made in the manner of section 4.2, this time comparing AAD patients with and without the *PTPN22* risk allele. Figure 4.9 shows the gene expression of the 11 chosen autophagy genes in AAD patients which are heterozygous or homozygous for the risk allele (grey columns), and AAD patients with the non-risk allele (white columns). Comparing the groups deemed a significant change in the gene expression of *ATG5*, *ATG12* and *GABARAP* in patients with the risk allele compared with patients with the non-risk allele. In contrast, *ULK1*, *ULK2*, *ATG4B*, *ATG7*, *ATG9A*, *BECN1*, *PIK3C3* and *MAP1LC3B* showed no significant change between the groups.

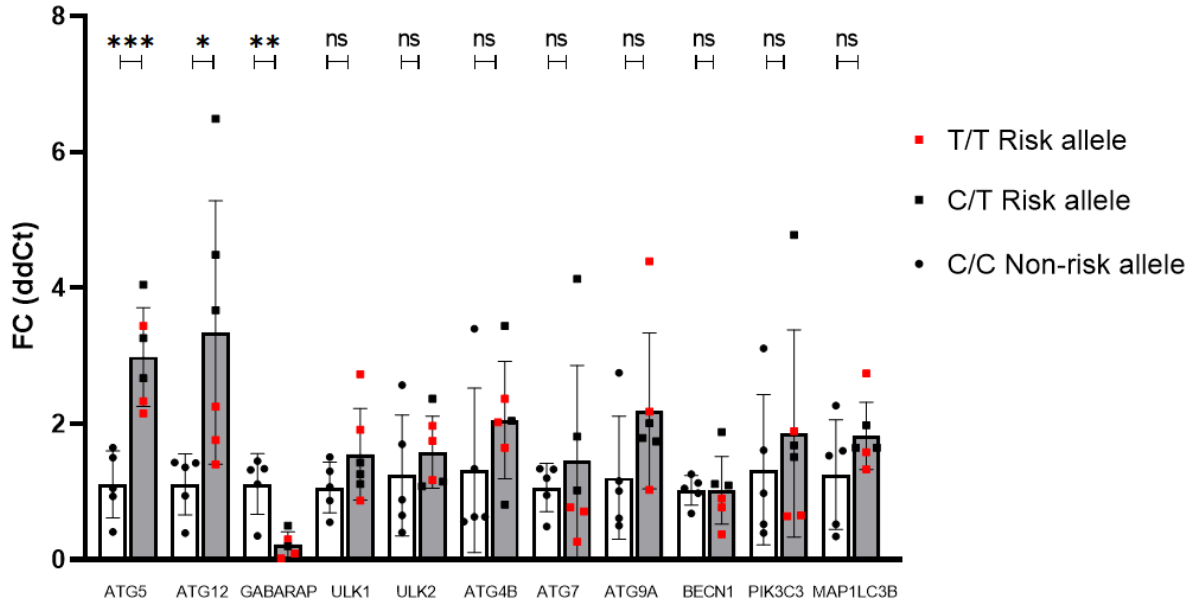


**Figure 4.9: Autophagy gene expression in AAD patients with and without the PTPN22 risk allele.** Relative to their non-risk allele (C) counterparts, AAD patients with the risk allele (T) showed higher expression of *ATG5* and *ATG12*, and lower expression of *GABARAP* in whole blood. Samples from nine patients were used for each gene in triplicates for each reaction. The $C_t$s assessed via real-time qPCR and presented by QuantStudio™ Design & Analysis Software were extracted to Excel, where fold change (FC) was calculated by using the $2^{-\Delta\Delta Ct}$-method with GAPDH as the reference gene. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, ns = not significant, unpaired T-test with Welch's correction for unequal variance.

## 4.4 PTPN22 knockdown positively associated with ATG5 in THP-1 cells

In order to test whether there is any causality between *PTPN22* and the changed expression of *ATG5*, *ATG12* and *GABARAP*, knockdown (KD) of *PTPN22* was carried out with siRNA. As all of these have low immune cell specificity and a balanced distribution among innate and adaptive cells[117], the human monocytic cell line THP-1 was chosen and deemed to be satisfactory for the transfection experiment.

In addition to maintaining the cells in their preferred media conditions with 10% FBS post-transfection, they were also kept with 1% FBS in order to induce autophagy and see whether a noticeable effect was displayed. Figure 4.10 shows cells maintained with 10% FBS post-transfection in a) and 1% FBS in b). Comparisons between samples transfected with *PTPN22* siRNA and control siRNA were this time made via unpaired T-tests without Welch's correction, as equal sample sizes and standard deviations are assumed in consequence of being derived in the same concentrations, and from clones. The results display successful KD of *PTPN22* in both 4.10a) and b), and a significant increase in *ATG5* expression in THP-1 cells kept with 10% FBS post-transfection of *PTPN22* siRNA. No significant change was found in the expression of *ATG5* in cells kept with 1% FBS, neither in *ATG12*

nor *GABARAP* in either of the conditions.

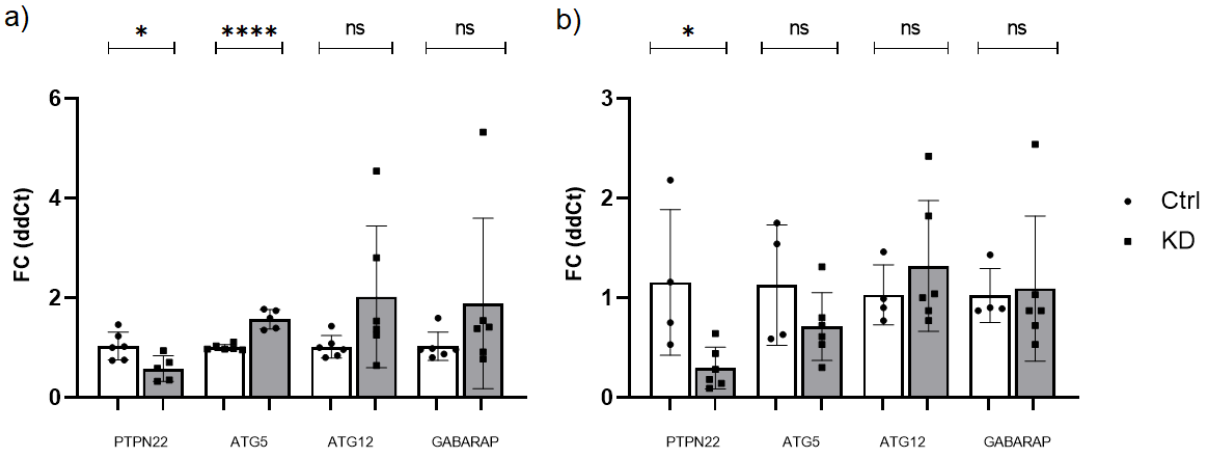


**Figure 4.10: ATG5 is upregulated in PTPN22 siRNA transfected THP-1 cells incubated with 10% FBS.** PMA-differentiated THP-1 cells were treated with either control (Ctrl), or PTPN22 siRNA (KD) combined with Lipofectamine™ 2000 according to its protocol [118]. After 4h incubation with combined reagents, the media solution was replaced with fresh media containing **a)** 10% FBS or **b)** 1% FBS before further incubation for 24h. The cells were then assessed for gene expression via RNA extraction and real-time qPCR for the chosen genes, by using the $2^{-\Delta\Delta Ct}$-method on the $C_t$s presented by QuantStudio™ Design & Analysis Software extracted to Excel, with GAPDH as the reference gene. * = $p \leq 0.05$, **** = $p \leq 0.0001$, ns = not significant, unpaired T-test with equal variance assumed.

# 5. Discussion

## Summary

In the present study, the autophagy-related transcripts *ULK1*, *ULK2*, *ATG9A* and *ATG12* were found to be higher expressed in whole blood of patients with AAD compared to healthy controls, whilst *GABARAP* was found to be lower expressed. This was found to be independent of the amounts of monocytes and neutrophils in the blood. The *PTPN22* risk allele (R620W) was shown to be positively associated to *ATG5* and *ATG12*, and negatively associated to *GABARAP*, whilst showing no association with *ULK1* and *ULK2*. The causality between the risk allele and these genes were further studied by silencing *PTPN22* in a monocyte cell line with siRNA, which confirmed a positive association with *ATG5*. An overview of the findings is presented in Table 5.1.

**Table 5.1. Autophagy genes found to have significant changes in the study.** Blank fields indicate that no significant changes were found.

| Gene | Patient vs Control | PTPN22 Risk vs Non-risk | PTPN22 KD |
|---|---|---|---|
| ULK1 | ↑ | | |
| ULK2 | ↑ | | |
| ATG5 | | ↑ | ↑ |
| ATG9A | ↑ | | |
| ATG12 | ↑ | ↑ | |
| GABARAP | ↓ | ↓ | |

## 5.1 Altered autophagy genes in blood of patients with AAD

Of the 11 autophagy-related genes tested for, five were altered in the blood of patients with AAD; *ULK1*, *ULK2*, *ATG9A* and *ATG12* upregulated, and *GABARAP* downregulated (Table 4.4). Carrying individual roles in the autophagy-machinery, ULK1 and its isoform ULK2 are responsible for its initiation, ATG12 and GABARAP conjugate in each of their own complexes which nuclearize and expand the phagophore vesicle, and ATG9A scrambles the phospholipids required to form the membrane of what becomes the autophagosome. All vital player in canonical autophagy, they are part of the major degradation pathway which delivers invading pathogens, damaged organelles, aggregated proteins, and other macromolecules from the cytosol to the lysosome for bulk degradation [119][120].

As the research conducted on autophagy in AAD is limited, one can only speculate upon the alteration of these genes in regard to diseases with similar characteristics. A growing number of studies have connected dysregulated autophagy to autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS), Crohn's disease and SLE [98][99]. Comparing gene expression in whole blood from patients with MS compared to healthy controls found *ULK1* and *ULK2* be *upregulated*, *ATG9A* to be downregulated, and no significant difference in levels of *ATG12* and GABARAP[121]. PBMC from patients with SLE have increased *ATG12*, and patients with Parkinson's (which is a partial autoimmune disease) have increased *GABARAP*[122][123][124].

Although mRNA levels of autophagy-related genes do not necessarily correspond with autophagic activity, a trend is apparent. To look into whether the whole blood autophagy gene expression levels were related to altered ratios of monocytes or neutrophils in AAD patients compared to healthy controls, their levels were recorded via flow cytometry.

## 5.2 Ratios of monocytes and neutrophils remain unaltered in patients with AAD

Analysing the counts of monocytes and neutrophils in the same age and sex group for patients with AAD and healthy controls showed no alteration in their relative abundance. Being an indicator for inflammation, perturbed ratios have been associated with various autoimmune diseases such as, MS, IBD, RA and SLE: elevated levels of both neutrophils and monocytes in patients with MS, RA and SLE, and elevated levels of CD16+ monocytes in IBD and RA [125][126][127][128].

Since autophagy genes are expressed in different amounts depending on the cell type [116][117], elevated or decreased gene expression measurements may be direct consequences of different cell counts for the different subsets in patients and healthy controls. This is usually solved for by looking at gene expression in separated subsets of cells from whole blood, comparing results from the same subsets between patient and control, instead of comparing whole blood to whole blood. However, the analysis of data obtained by their comparison through flow cytometry shows no alteration in ratios of monocytes or neutrophils to CD45+ leukocytes between patient and control, indicating roughly equivalent amounts of total RNA, and that the altered expression of autophagy genes in patients with AAD stems from another cause. Such a cause could be an alteration in their abundance of T cells: known to have a wide expression of *ATG* genes, an increased abundance of this subset would skew the results obtained in this experiment. Looking at this subset in the future would shed light on the accuracy of this study.

## 5.3 The PTPN22 risk allele is positively associated with ATG5 and ATG12, and negatively associated with GABARAP

Comparing the expression of the 11 selected autophagy genes, patients with AAD having the *PTPN22* risk allele (R620W) displayed a positive association to *ATG5* and *ATG12*, and a negative association to *GABARAP*, compared to AAD-patients without the risk allele. This polymorphism of *PTPN22* is a well-known associate with autoimmune disease, having been associated with SLE, RA, T1D, and recently AAD[75][129]. In addition to rendering the protein less efficient in regulating T cell activation, which causes their increased proliferation and cytokine secretion, loss of *PTPN22* is also associated with enhanced NF-κB and MAPK activation, which initiate the secretion of cytokines recruiting ATG16L1[130][131][100]. ATG5 and ATG12 are tightly connected to ATG16L1 in nuclearization of the membrane which becomes the autophagosome, where ATG12 covalently binds to ATG5, before conjugating with ATG16L1. This complex binds to WIPI2, which brings ATG5-ATG12-ATG16L1 to pre-autophagosomal structures where it allows another complex, consisting of GABARAP form II and PE, to be incorporated into their membrane[120] (Figure 1.3).

The negative association of *GABARAP* in regard to *PTPN22* risk allele found in this study is argued by Spalinger et al., who found the GABARAP orthologue LC3B to increase in response to loss of *PTPN22* [132]. Both GABARAP and LC3 are members of the ATG8 family, associated with biogenesis and sealing of the autophagosome membrane, as well as escorting cargo to the vesicle by interacting with cargo receptors carrying ATG8-interacting motifs. Although the ATG8s are not essential in the forming and closing of the autophagosome, they are a crucial part of the final step of autophagy: fusion with the lysosome [133]. Routinely used as an indicator of autophagy flux [134], *GABARAP* being downregulated in patients with AAD could therefore indicate a reduction in autophagy despite the increase in *ATG5* and *ATG12*, as their efforts are nullified in consequence of the autophagosome not being able to fuse with a lysosome for degradation. This would although require further studies in other to confirm.

## 5.4 Silencing PTPN22 upregulates ATG5

Testing the causality of autophagy gene regulation by PTPN22 in vitro confirmed the association with *ATG5*, but not *ATG12* and *GABARAP*, in *PTPN22*$^{KD}$ THP-1 cells. Incubating adhered monocytes with 10 % and 1 % FBS post-transfection of siRNA targeted towards *PTPN22* showed successful knockdown of *PTPN22* for both conditions. Interestingly, only cells incubated in 10 % FBS post-transfection showed a significant change in their expression of *ATG5*, and not cells incubated in starvation conditions.

Both innate and adaptive immune systems have shown to be regulated by ATG5-mediated autophagy. In the innate, ATG5 regulates macrophage polarization by altering their extent of inflammation via M2 induction, halting the inflammatory cytokine secretion of M1[135]. ATG5 suppresses NF-κB and MAPK activation by interacting with MyD88 [136], an adaptor molecule which activates the innate signalling cascade via TLR and the IL-1 receptor, and indirectly activates neutrophils, by inducing the extrusion of mitochondrial contents when present with LPS which provokes an inflammatory response [137]. In the adaptive system, ATG5 is indirectly responsible in lymphocyte activation by promoting their interaction with APCs through the delivery of pathogen-derived peptides to MHC-II molecules via autophagy [83]. It also directly regulates them, as the deletion of *ATG5* in $CD4^+$ and $CD8^+$ T cells have shown to fail to proliferate by TCR stimulation, the same case being for *ATG5*-deleted B cells which fail to mature properly [103].

Aberrant *ATG5* has been indicated in a plethora of diseases [138]. To mention some of the immune-related; asthma [139], diabetes[140] and melanoma [141] have all been related to altered levels of *ATG5*. Increases in *ATG5* have been shown in Alzheimer's disease [142] and MS [143], and links towards ATG5 interplay with the non-canonical autophagy pathway LC3-associated phagocytosis

(LAP) have been shown in the pathogenesis of SLE [144]. Although no significant change was detected in *ATG5* levels comparing AAD patients with healthy controls in the present study, changes in PTPN22 showed upregulation of *ATG5* in both patients with the R620W risk allele and *PTPN22*$^{KD}$ THP-1 cells in vitro. This indicates that the upregulation of *ATG5* is not a direct consequence of AAD in this patient group, but rather is connected to aberrant function in *PTPN22*.

## 5.5 Limitations of the study

The experiments conducted in this study have limitations both in the analyses of whole blood and human monocytic cell line THP-1. Studying whole blood puts all cell types under the same umbrella, not conveying the differences in function and gene expression between them. Whether the data extracted from these samples are representative for the expression of autophagy genes, and not skewed by the up- or downregulation of specific cell types expressing them is therefore not known. Another limitation in the data derived from the whole blood samples is the lack of proteomics, as only the RNA of genes was analysed, and it is not known if this reflects their products after protein translation.

Studying the effects of *PTPN22*$^{KD}$ in THP-1 cells was limited in the nature of the siRNA knockdown, only reducing the expression of the gene and not completely eliminating it. Indeed, only a certain percentage of *PTPN22* expression was reduced. The activation of autophagy via FBS serum starvation was also limited in the sense that no tests were carried out to confirm that autophagy had taken place. This compromises the validation of data gained in this experiment, depriving any eventual conclusions related to autophagy.

## 5.6 Future perspectives

To overcome the limitations mentioned above, the use of single cell RNA sequencing, proteomics, gene knockout and autophagy assays may be carried out. Single cells can be FACS isolated based on their cell specific markers, allowing for the studying of gene expression in individual cell populations contrary to all at the same time, which was the case in the present study. Such studies may involve proteomic techniques such as western blot or mass spectrometry to determine the presence of autophagy proteins individually, or by looking for the presence of GABARAP/LC3 to determine the overall autophagy flux in patients with AAD. This may also be conducted via specialized autophagy assays which stain LC3 products, allowing for their precise quantification [145].

THP-1 cells may have *PTPN22* fully knocked out by using CRISPR/Cas9, which can also be utilized to create separate populations which are either homozygous for the risk allele or the major allele. One

may thus further analyse the expression of autophagy genes in the separate populations for a more accurate assessment of their regulation by PTPN22 than what was conducted in the present study. Using the proteomic techniques mentioned, the measurement of the proteins translated from autophagy genes would then also be able to be assessed in vitro, determining further causality and allowing for their placement in a broader immunological perspective.

Such a perspective could be to look at co-stimulatory molecules as prospects of regulation via altered autophagy. As CD80 and CD86 have been shown to be inducted with autophagy[106], it is possible that increased autophagy in patients with AAD could induce an increase in the levels of co-stimulatory molecules activating the proliferation of $CD4^+$ $T_h$ cells, which are involved in the onset of the disease. This would lead to the idea of stratifying AAD patients with personalized medicine towards the *PTPN22* risk allele as a possible treatment.

## 5.7 Concluding remarks

The involvement of autophagy stretches throughout the vast and complex landscape which is the maintenance of the immune system: regulating its compartments for proper function, but also being regulated itself by the same compartments in a two-way symbiosis. In the present study, transcripts of autophagy genes *ULK1*, *ULK2*, *ATG9A*, *ATG12* and *GABARAP* were found to be altered in the whole blood patients with AAD. As aberrant autophagy has been found to be present in a number of autoimmune diseases, the alteration of these genes may indicate the same to be true in AAD. No conclusions may however be drawn as the sample sizes were limited and no proteomics studies were carried out to confirm these findings at a protein level.

Further, a regulator of autophagy and many other immune functions, *PTPN22* was associated with an alteration in *ATG5*, *ATG12* and *GABARAP* expression levels in AAD patients with the *PTPN22* (R620W) risk allele, compared to AAD patients with the non-risk allele. Although the validation of a study with such few samples is hard to recognize, the association with ATG5 was further confirmed in $PTPN22^{KD}$ THP-1 cells, bringing forth the interesting field which is the interaction between the two titans of immune regulation. Indeed, both autophagy and PTPN22 have been studied intensely, although studies on their interaction in between are few. Producing more research on the interplay between them could shed light on their role in the grander scale of autoimmune disease, such as is the case for Addison's autoimmune disease.

# 6. References


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# Appendix

**Table A.1: Patients and controls used in study.** Some samples had been genotyped for PTPN22 previously by the research group as part of a genome wide association study. These are marked T and C for the minor and major allele, respectively. All patients were donors to the ROAS study.

| Ref ID. | Sex | Age | Patient/control | PTPN22 | Usage |
|---|---|---|---|---|---|
| GC1 | Female | 53 | Control | Unknown | Autophagy gene expression |
| GC2 | Female | 55 | Control | Unknown | |
| GC3 | Male | 50 | Control | Unknown | |
| GC4 | Female | 60 | Control | Unknown | |
| GC5 | Female | 55 | Control | Unknown | |
| GC6 | Male | 62 | Control | Unknown | |
| GC7 | Female | 63 | Control | Unknown | |
| GC8 | Female | 54 | Control | Unknown | |
| GC9 | Female | 51 | Control | Unknown | |
| GC10 | Female | 56 | Control | Unknown | |
| GC11 | Female | 62 | Control | Unknown | |
| GP1 | Female | 78 | Patient | T/T | |
| GP2 | Female | 64 | Patient | T/T | |
| GP3 | Female | 59 | Patient | T/T | |
| GP4 | Female | 62 | Patient | C/C | |
| GP5 | Female | 72 | Patient | C/T | |
| GP6 | Female | 70 | Patient | C/T | |
| GP7 | Female | 53 | Patient | C/T | |
| GP8 | Female | 64 | Patient | C/C | |
| GP9 | Female | 61 | Patient | C/C | |
| GP10 | Female | 76 | Patient | C/C | |
| RC1 | Female | 54 | Control | Unknown | Assessing relative abundance of monocytes and neutrophils |
| RC2 | Male | 41 | Control | Unknown | |
| RC3 | Female | 60 | Control | Unknown | |
| RC4 | Male | 52 | Control | Unknown | |
| RC5 | Female | 32 | Control | Unknown | |
| RC6 | Female | 21 | Control | Unknown | |
| RC7 | Male | 55 | Control | Unknown | |
| RC8 | Male | 62 | Control | Unknown | |
| RC9 | Female | 55 | Control | Unknown | |
| RC10 | Male | 55 | Control | Unknown | |
| RC11 | Male | 51 | Control | Unknown | |
| RC12 | Female | 63 | Control | Unknown | |
| RCP1 | Female | 65 | Patient | Unknown | |
| RP2 | Female | 35 | Patient | Unknown | |
| RP3 | Female | 53 | Patient | Unknown | |
| RP4 | Female | 68 | Patient | Unknown | |
| RP5 | Female | 23 | Patient | Unknown | |
| RP6 | Male | 44 | Patient | Unknown | |
| RP7 | Male | 66 | Patient | Unknown | |
| RP8 | Male | 59 | Patient | Unknown | |
| RP9 | Female | 53 | Patient | Unknown | |
| RP10 | Male | 17 | Patient | Unknown | |
| RP11 | Male | 55 | Patient | Unknown | |
| RP12 | Male | 65 | Patient | Unknown | |

| | | | | | |
|---|---|---|---|---|---|
| RP13 | Male | 41 | Patient | Unknown | |
| RP14 | Male | 60 | Patient | Unknown | |

**Table A.2: Concentrations of isolated RNA and amounts loaded with PowerTrack SYBR Green.**

| Sample Ref. | Concentration (ng/µL) | A260/A280 | Amount used (µg) | Source |
|---|---|---|---|---|
| GC1 | 47.53 | 2.08 | 0.5 | Patients with AAD and healthy controls |
| GC2 | 85.58 | 2.19 | 0.5 | |
| GC3 | 50.77 | 2.09 | 0.5 | |
| GC4 | 82..86 | 2.07 | 0.5 | |
| GC5 | 67.71 | 2.18 | 0.5 | |
| GC6 | 82.19 | 2.06 | 0.5 | |
| GC7 | 48.82 | 2.14 | 0.5 | |
| GC8 | 65.36 | 2.06 | 0.5 | |
| GC9 | 54.75 | 2.10 | 0.5 | |
| GC10 | 47.53 | 2.08 | 0.5 | |
| GC11 | 81 | 2.20 | 0.5 | |
| GP1 | 98.60 | 2.09 | 0.5 | |
| GP2 | 31.46 | 2.15 | 0.5 | |
| GP3 | 183.86 | 2.06 | 0.5 | |
| GP4 | 80.89 | 2.12 | 0.5 | |
| GP5 | 93.272 | 2.02 | 0.5 | |
| GP6 | 93.06 | 2.08 | 0.5 | |
| GP7 | 70.76 | 2.07 | 0.5 | |
| GP8 | 46.04 | 2.13 | 0.5 | |
| GP9 | 173.49 | 2.08 | 0.5 | |
| GP10 | 58.35 | 2.08 | 0.5 | |
| PTPN22 siRNA, 1% FBS | 97.34 | 2.04 | 1.0 | Human monocytic cell line THP-1 |
| | 104.83 | 2.07 | 1.0 | |
| | 118.51 | 2.06 | 1.0 | |
| | 123.35 | 2.08 | 1.0 | |
| | 101.62 | 2.09 | 1.0 | |
| | 93.85 | 2.07 | 1.0 | |
| NC siRNA, 1% FBS | 124.98 | 2.07 | 1.0 | |
| | 93.28 | 2.07 | 1.0 | |
| | 117.26 | 2.06 | 1.0 | |
| | 94.26 | 2.03 | 1.0 | |
| PTPN22 siRNA, 10% FBS | 126.22 | 2.05 | 1.0 | |
| | 133.40 | 2.07 | 1.0 | |
| | 100.94 | 2.01 | 1.0 | |
| | 110.17 | 2.08 | 1.0 | |
| | 103.02 | 2.04 | 1.0 | |
| | 100.13 | 2.06 | 1.0 | |
| NC siRNA, 10% FBS | 115.00 | 2.06 | 1.0 | |
| | 111.13 | 2.06 | 1.0 | |
| | 114.48 | 2.08 | 1.0 | |
| | 121.77 | 2.07 | 1.0 | |
| | 92.14 | 2.05 | 1.0 | |
| | 101.83 | 2.03 | 1.0 | |