Pharmacological treatment of the T – cell receptor signalling pathway in autoimmune Addison's disease

A pilot study with abatacept (CTLA4-Ig)

Master thesis in Pharmacy

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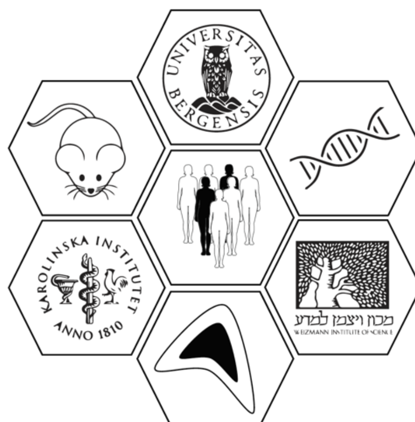

Abstract

Autoimmune Addison's disease (AAD) is characterized by an autoimmune destruction of hormone producing cells in the adrenal cortex. The pathogenesis of the disease involves autoantibodies and autoreactive T-cells against 21-hydroxylase, an enzyme involved in the production of adrenal gland hormones. No cure exists for the disease and these patients are dependent on life-long replacement therapy causing reduced quality of life and a higher risk of death. It is therefore important to develop new treatment options that can improve patients' quality of life, restore self-tolerance and possibly reverse the autoimmune reaction. *CTLA4,* encoding a vital inhibitory molecule involved in T-cell signalling, is a possible susceptibility gene for disease development. Abatacept is a soluble CTLA4 molecule, which have been approved for use in the treatment of the autoimmune disease rheumatoid arthritis, blocking a co-stimulatory signal necessary for complete T-cell activation, thus reducing proliferation and the production of cytokines. Conflicting results have been shown in studies examining the effect of abatacept on regulatory T-cells (Tregs) in autoimmune disorders.

We hypothesized that treatment with abatacept (CTLA4-Ig) in AAD patients could increase the number of Tregs and suppress the autoimmune response by affecting the inhibitory function of CTLA4. To explore this, we developed a method for screening the effect of this drug on T-cells in AAD patients and healthy controls at the KG Jebsen Center for Autoimmune Diseases. The methodology included cell culture of peripheral blood mononuclear cells (PBMC), flow cytometry, quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA).

We succeeded in developing an *in vitro* method on primary cells that can be used to screen the effect of abatacept on T-cells in patients with AAD. Although our study is considered to be preliminary due to a low number of patients, our results indicate that abatacept affects proliferation and the fraction of live cells in both patients and controls, and had a varying effect on the Treg population in both cohorts. IFN-γ levels were higher in AAD patients than in controls both before and after drug treatment, but both groups showed decreased production of IFN-γ as a consequence of abatacept. Future studies should include a higher number of individuals to be investigated in order to reveal treatment-specific differences between patients and controls in regard to abatacept treatment.

Abbreviations

AAD	Autoimmune Addison's Disease
ACTH	Adrenocorticotropic hormone
AIRE	Autoimmune regulator
APC	Antigen presenting cell
APS-1 or 2	Autoimmune Polyendocrine Syndrome Type 1 or 2
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxy fluorescein diacetate succinimidyl ester
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FasL/FASLG	Fas ligand/Fas Ligand Gene
FCS	Forward light scattering
FOXP3	Forkhead box P3
GWAS	Genome wide association study
HRP	Horseradish peroxidase
ICOS	Inducible T-cell co-stimulator
IFN-γ	Interferon γ
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, endocrinopathy, X-linked
MHC	Major histocompatibility complex
mTEC	Thymic medullary epithelial cell
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered saline
(q)PCR	(Quantitative) Polymerase Chain Reaction
REK	Regional committees for medical and health research ethics
RIN	RNA integrity number
SSC	Side light scattering
TCR	T-cell receptor
TGF-β	Transforming Growth Factor β
Th cells	T helper cells
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Treg(s)	Regulatory T-cell(s)

1. Introduction

1.1. The immune system

The immune system is the human body's main defence against threats from the outside and consists of two main parts; innate and adaptive immunity (1). When a pathogen (bacteria, virus etc.) enters the body, it meets its first obstacle in the innate immune system (2). This is made up of chemical and physical barriers, acute phase proteins, the complement system and different types of cells, such as dendritic cells and macrophages, that can respond to the presence of pathogen-associated molecular patterns (PAMPs) (2, 3). Looking at the aspect of time, the non-specific innate response sets in almost immediately after encountering a pathogen, which is of major importance as many bacteria can double in number within a short amount of time (2, 4). The components of the innate immune system do not generate immunological memory and some of its main missions are to detect foreign substances and produce signalling molecules (cytokines, chemokines) that will attract other immune cells to an infection site and alert the adaptive immune system (5, 6). An adaptive response is then initiated and consists of a humoral and a cellular branch, mediated by B- and T-lymphocytes, respectively (7).

1.2. B-cells

B-cells develop and become fully mature in the bone marrow (1). B-cells are important for the function of T-cells, as they can act as antigen presenting cells (APC) (8), while T-cells contribute to B-cell function by stimulating them to differentiate and proliferate (9). Unlike T-cells, B-cells do not depend upon antigen presentation, as they can eliminate extracellular pathogens on their own through the production of antibodies (1), which starts after the activated B-cells have differentiated into antibody producing and secreting plasmablasts and/or plasma cells (10). Also, these antibodies have a dual role as they will work to prevent development of an infection, but they could also cause tissue damage in organ transplants and autoimmunity (11). After eliminating a foreign disease-causing substance, a fraction of activated B-cells will turn into memory B-cells, and if the host is infected by the same pathogen at a later point in life, the immune response will be much faster (12).

1.3. T-cells

T-lymphocytes develop from hematopoietic stem cells in the bone marrow, before the T-cell progenitors travel to the thymus to continue their development (1). There are two different subgroups of conventional T-cells; cluster of differentiation (CD) 4 T-helper (Th) cells (further

divided in the effector T-cells Th1, Th2 and Th17) that produce cytokines important for the activation of B-cells and for the function of CD8 T-cells, and CD8 cytotoxic T-cells that can directly eliminate cells infected by intracellular pathogens, such as viruses (13-17) (Fig. 1). T-cells are not able to recognize foreign substances on their own; fragments of antigens have to be presented bonded to major histocompatibility complex molecules (MHC) on an APC, before the T-lymphocytes can be involved in an immune response (18).

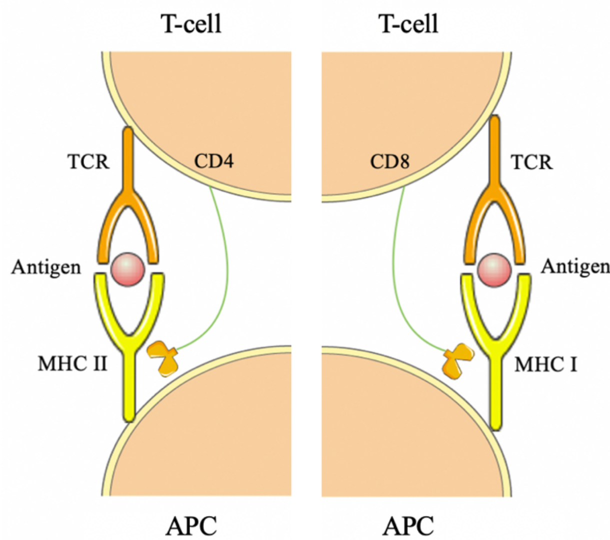

Figure 1: Antigen presentation and co-receptors. *Presentation of antigen through MHC-molecules is required for the initiation of the T-cell immune response. CD4⁺ cells recognize antigens presented by MHC II, while CD8⁺ cells respond to antigens presented by MHC I. This figure contain elements from Servier Medical Art by Servier, 2020.*

Also important for T-cell function is the T-cell receptor (TCR) (19). For the majority of T-cells it consists of two chains, α and β, both of which have a constant, variable, diversity and joining region, and rearrangements of these make it possible for T-cells to respond to almost every pathogen threatening the host (1, 19). Rearrangement of the TCR is important in the formation of $CD4^+$ and $CD8^+$ cells in the thymus, ensuring MHC class restriction (19). Signalling through the TCR is also dependent on the formation of a complex with CD3, containing a cytoplasmic part necessary for downstream signalling and activation (20-22), but also require interaction with a co-receptor; CD4 for the helper cells responding to MHC class II molecules and CD8 for cytotoxic cells responding to MHC class I molecules (23, 24) (Fig. 1).

1.4. Thymic development of T-lymphocytes

The thymus is a lymphoepithelial organ located in front of the heart, but behind the chest bone, and is the main site for T-cell development (7). After migrating from the bone marrow to the thymus, early T-cell precursors are in a double negative ($CD8^-CD4^-$) state, before signaling through a pre-TCR complex, which is important for maturation of the cell strain, takes the progenitor cells/thymocytes to a double positive ($CD8^+CD4^+$) state (25-27) (Fig. 2). The next step is for the double positive $CD8^+CD4^+$ thymocytes to develop into either $CD4^+$ or $CD8^+$ single positive cells, a process that rely on interactions with MHC class I or MHC class II

molecules (28) (Fig. 1). Further, positive selection in the thymic cortex will select the cells that bind self-MHC class I or II, to become single-positive thymocytes (29) (Fig. 2). The positive selected thymocytes then migrate to the thymic medulla, where negative selection will lead to the deletion of thymocytes that react to strongly to self-peptides presented by MHC class I or II (29). Negative selection is an important step in T-cell development that has been shown to happen through programmed cell death, also known as apoptosis, and might be caused by the interaction between Fas and its ligand (FasL) (30-32).

A protein of major importance in negative selection and the discrimination between self- and non-self is the autoimmune regulator (AIRE) (33). This protein can be found in thymic medullary epithelial cells (mTECs), where it controls the expression of peripheral tissue-restricted antigens, such as insulin, and present them to precursor T-cells (33). The importance of accurate negative selection is exemplified by a mutation in or lack of expression of *AIRE,* which leads to the development of autoimmune polyendocrine syndrome (APS) type 1 (34). This is a monogenic disorder characterized by the presence of two out of three components; Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis (34). It has also been shown that *AIRE* plays a role in the thymic formation of another subset of $CD4^+$ T-cells expressing Forkhead Box P3 (*FOXP3)*, called regulatory T-cells (Tregs) (35) (section 1.5). After completing their development in the thymus, naïve $CD4^+$, $CD8^+$ and Treg cells will relocate to the periphery and start "patrolling" the environment for invaders (36, 37) (Fig. 2).

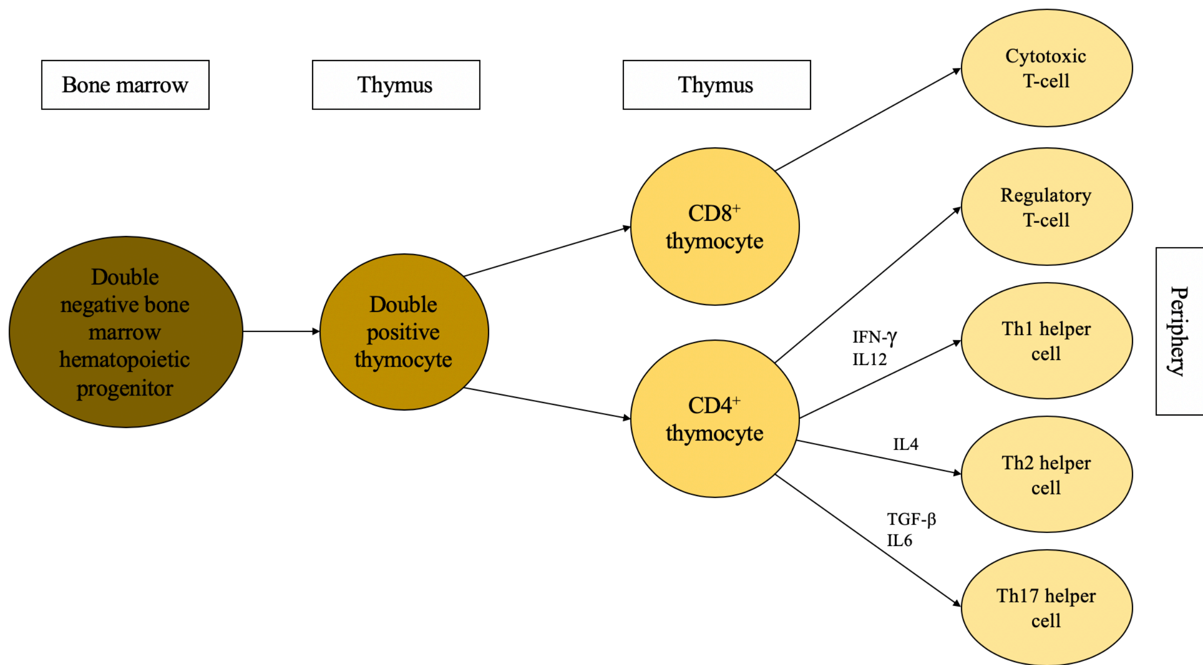

Figure 2: Development of T-cells. *Bone marrow stem cells migrate to the thymus and serve as double negative T-cell progenitors. Rearrangement of the TCR-β-chain gives rise to double positive thymocytes, which in turn becomes CD4⁺ or CD8⁺ single positive cells. These will further differentiate, in the periphery, to different subpopulations of T-cells dependent on cytokine production and secretion. IFN-γ and IL2 give rise to Th1 cells, IL4 causes differentiation into Th2 cells, while IL6 and TGF-β stimulates Th17 cell differentiation.*

1.5. Regulatory T-cells

Tregs are a subpopulation of $CD4^+$ T-lymphocytes that can control the effect of other immune cells and thus have a crucial function in the maintenance of self-tolerance (38, 39). Commitment to the Treg cell lineage is thought to depend on the strength by which the cell binds the self-peptide/MHC-complex in the thymus; too high affinity will trigger negative selection, low affinity will cause generation of conventional naïve T-cells and an affinity between these outer limits will give rise to a fraction of *FOXP3* expressing cells, known as thymic derived Tregs (tTregs) (40-42). Not all regulatory T-cells are formed in the thymus; a subtype known as peripheral Tregs (pTregs) arise from conventional $CD4^+$ T-lymphocytes outside the thymus (37). Precursors of both tTregs and pTregs depend upon signalling through CD28 (section 1.6) to become *FOXP3* expressing Tregs, for survival and for maintenance of homeostasis outside the thymus (43-45).

Different mechanisms as to how Tregs mediate suppression of the immune response have been suggested (37). There are at least four main pathways that these cells could follow: The first is controlled by, amongst others, cytotoxic T-lymphocyte associated protein 4 (CTLA4),

constitutively expressed by Tregs, through cell-cell-contact (37, 46). The function of CTLA4 will be described later (section 1.8). A second possible mechanism of action is caused by the constitutive expression of CD25 by Tregs, which make them a contender to effector T-cells in the race for the cytokine interleukin (IL) 2 (37). Further, it has been found that IL2, as for conventional T-cells, is important for the function and survival of Tregs (47-49). The third mechanism involves the production and secretion of cytokines, such as IL10 and transforming growth factor β (TGF-β), which will affect function, activation and the inhibitory properties of Tregs (37, 41). Finally, it has been suggested that Tregs have the ability to cause direct apoptosis, due to the production of granzymes (37, 50).

Tregs are one of the main characters among the peripheral tolerance mechanisms, working to protect the body against autoimmunity and also play an important role in limiting an unwanted excessive immune reaction (38). If the peripheral tolerance mechanisms fail, autoimmunity could arise either as a consequence of malfunctioning Tregs, reduced frequency of Tregs or due to the ability of conventional T-cells to resist Treg mediated suppression (51, 52). Malfunctioning Tregs can lead to development of autoimmunity, as mutations in the Treg key regulator *FOXP3* causes immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) (53, 54). The role of Tregs in self-tolerance and autoimmunity make them a potential target for therapy (55, 56).

1.6. T-cell activation

For a T-cell to be able to perform its duty in the immune response, it has to be activated (57). This process starts with the interaction of the TCR with the antigen-MHC-complex on the APC (57). The APCs present fragments of the antigen through MHC class I or II on their cell surface, which is then recognized by a CD8 or CD4 cell, respectively, and the first step in the activation process is completed (23, 24, 57) (Fig. 3, modified from (58)). For proliferation and differentiation to take place, a co-stimulatory signal is necessary (57). Furthermore, it has been shown that lack of co-stimulation will render the cell anergic, meaning that it is in a state of unresponsiveness (59). CD28 is a molecule on the T-cell surface that can provide such a co-stimulatory signal (60) (Fig. 3). CD28 will bind the co-stimulatory ligands CD80 and CD86 on the APC, and with the TCR-MHC/antigen binding already present, the T-cell is activated (43) (Fig. 3).

Activation leads to upregulation of the IL2-receptor α chain (CD25) on the T-cell surface, and the production and binding of IL2 stimulates further proliferation and differentiation (7). The effector function of the $CD4^{+}$ T-cells are decided by the production of cytokines by the APC; interferon γ (IFN-γ) and IL12 promotes differentiation into Th1 cells, IL4 promotes Th2 cells, while IL6 together with TGF-β have been shown to give rise to Th17 cells (61) (Fig. 2). IFN-γ plays a role in the immune response as it has been shown to increase the expression of MHC class II molecules on certain cells (62, 63). In addition, production of IFN-γ by Th1 cells will contribute to the activation of macrophages and the initiation of an antipathogenic response (64).

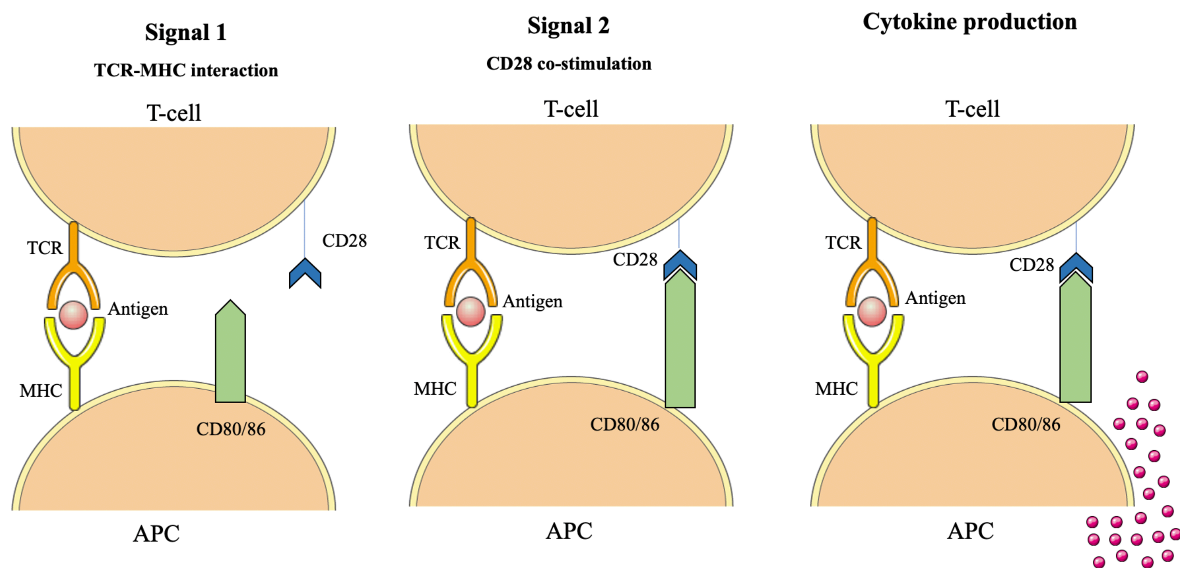

Figure 3: Activation of CD4 T-cells require ligation of both the TCR and CD28. *Signal 1 shows binding of TCR to the antigen-MHC class II-complex on the APC. Signal 2 is co-stimulatory, and lack of this signal will take the cell into a state of unresponsiveness. Cytokine production by the APC will determine the effector function of the CD4 T-cell. Further, the activated cells will expand, proliferate and initiate an immune response against a pathogen. Modified from Gutcher et al., 2007 (58). This figure contain elements from Servier Medical Art by Servier, 2020.*

1.7. Cellular markers of regulatory and conventional T-cells

Tregs are characterized by a set of intra- and extracellular markers, that can be used to characterise these cells by flow cytometry (65). Some of these markers, and conventional T-cells markers, will be briefly described below. Flow cytometry will be described in section 3.4.

CD3

CD3 is located on the surface of T- cells and plays an important role in T-cell signalling and activation (20). It forms a complex with the TCR, enabling generation of a signal necessary for

downstream signalling and activation (20, 22).

CD4

CD4 is a T-cell co-receptor located on the cell surface of T-helper cells, that can recognize fragments of antigen presented through MHC class II (23).

CD25

CD25, also known as the IL2 receptor α-chain, is upregulated on the cell surface of conventional T-cells after antigen stimulation and could be used as a marker of T-cell activation (66). It is also constitutively expressed by Tregs (37, 67) (section 1.5).

CD31

CD31 can be used to identify naïve T-cells due to the expression of this protein by T-cells that have recently left the thymus (68).

CD39

CD39 is a surface marker found present on Tregs and has been found to be particularly, but not exclusively, expressed by activated Tregs, and has been suggested to play a role in their suppressive function (65, 69, 70).

CD152

CD152, also known as CTLA4, is constitutively expressed by Tregs, and involved in one of the proposed mechanisms as to how these cells can mediate suppression of the immune response (37, 46) (section 1.5). CTLA4 will be further described in section 1.8.

CD304

The cell surface located CD304, also known as Neuropilin-1, has been suggested as a marker of Tregs (71). It has also been shown as a possible T-cell activation marker, and not as a distinct Treg marker (72). Two studies have shown that CD304 can be used to discriminate between tTregs and pTregs *in vivo* using animal models (73, 74).

CD45RA

CD45RA is a cell surface marker that can be used to separate naïve/resting Tregs and T-cells from those that have been activated (65, 75, 76).

Helios

Helios is another intracellular marker of Tregs (65). Helios is expressed by Tregs formed in the thymus, and within the peripheral tTreg population there are both Helios positive and negative cells with suppressive properties (77, 78).

FOXP3

Another intracellular marker is FOXP3, a transcription factor indispensable in the thymic development of Tregs (54). The intracellular expression of FOXP3 by Treg makes it possible to discriminate them from activated conventional T-cells, hence an essential marker for Tregs (65, 79).

1.8. CTLA4

After T-cell activation the protein receptor CTLA4 is expressed on the cell surface, together with CD28 (80). It has been suggested that its expression is induced by signalling through the TCR and co-stimulation through CD28 (81, 82). CTLA4 "competes" with the latter molecule on ligating CD80/CD86 on the APC, and will "win" due to higher affinity for the ligands (83, 84). Unlike CD28, the binding of CTLA4 provides a co-inhibitory signal, that will cause inhibition of T-cell activation and as a consequence halt the immune response (83, 85, 86) (Fig. 4, modified from (87)). The inhibitory properties of CTLA4 have been confirmed by experiments involving CTLA4-deficient mice, who develop lymphoproliferative disorders after a short period of time (88) and it has also been shown that CTLA4 is important for the suppressive role of *FOXP3*-expressing Tregs (89). Mutations in *CTLA4* have been found in humans, leading to, amongst others, lymphoproliferation and hypogammaglobulinemia (90). The inhibitory properties of CTLA4 makes it a promising target in the treatment of certain types of cancer, and agents blocking CTLA4, also called check-point inhibitors (e.g. Ipilimumab) are now widely used in the treatment of cancer (91). A known side effect of these drugs is the development of autoimmune disease, including Addison's disease or primary adrenal insufficiency (92, 93), which will be discussed in detail later.

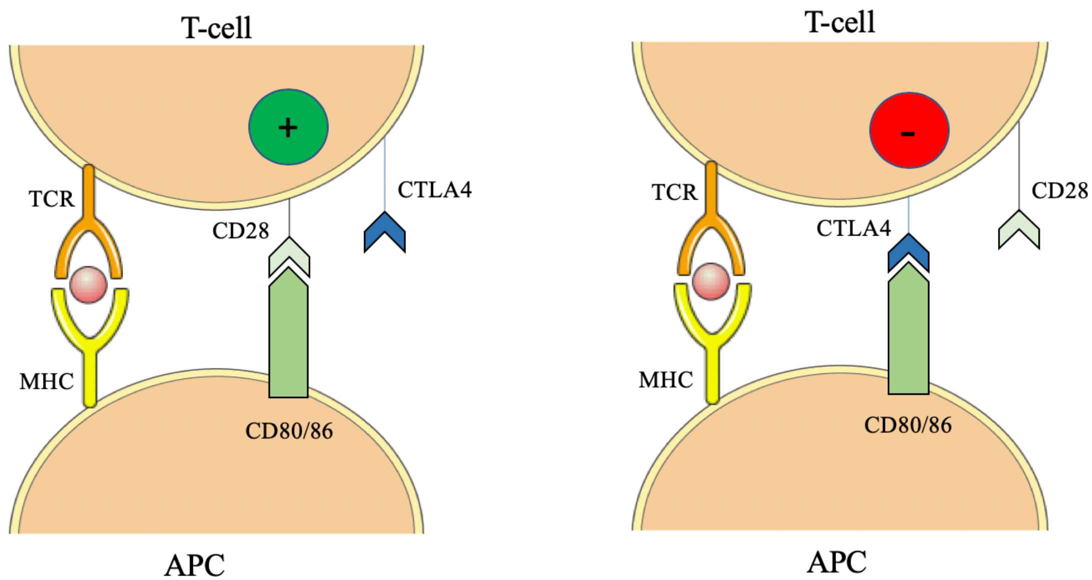

Figure 4: CTLA4 provides a co-inhibitory signal resulting in decreased T-cell activation. *T-cell activation depends on signalling through the TCR and ligation of CD28. After activation, CTLA4 is upregulated on the T-cell surface. CTLA4 will bind CD80/86 on the APC with higher affinity than CD28 and has an inhibitory effect on the T-cell. This leads to decreased T-cell activation, proliferation and differentiation. The result is a decreased immune response. The figure contains elements from Servier Medical Art by Servier, 2020. Modified from Lacouture et al., 2014 (87).*

There are several ways by which CTLA4 could exert its inhibitory function, in addition to "competing" with CD28 (94). One suggested mechanism is that CTLA4 prevents ligation of CD28 by removing CD80/CD86 from the APC in a process known as trans-endocytosis (95). Another suggestion is that CTLA4 has the ability to act as a modulator of the threshold required for signalling through the TCR and activation of T-cells (96, 97). Further, CTLA4 has been suggested to cause decreased expression of CD80/CD86 through the production of TGF-β and/or IL10, and to initiate generation of the enzyme indoleamine-2,3-dioxygenase by binding CD80/CD86 on dendritic cells, leading to reduced levels of tryptophan, both of which cause a downregulated T-cell response (83, 94, 98). CTLA4 has also been suggested to affect phosphorylation of the CD3 zeta chain in the TCR-complex (99), but this theory has been questioned as other studies have shown the opposite (100). In addition, polymorphisms in the *CTLA4* gene have been associated with susceptibility for development of autoimmune disease, where CTLA4 acts as a negative modulator by suppressing the immune response (94, 101, 102).

1.9. Autoimmune Addison's disease

Autoimmune Addison's disease (AAD) is an autoimmune disease leading to the destruction of cortisol producing cells in the adrenal cortex by the host's own immune system (103, 104).

AAD can exist as an isolated event or it could arise as a part of two different autoimmune syndromes; APS-1 or APS-2 (103) (section 1.4). AAD is considered a rare disease compared to for instance type 1 diabetes and autoimmune thyroid disease (103), with a prevalence of 144 per million in Norway (2008) (105). Most patients with AAD have circulating autoantibodies against the enzyme 21-hydoxylase, important in the biosynthesis of the hormones cortisol and aldosterone (105, 106). However, it is considered that the autoimmune destruction of cells in the adrenal cortex could be mediated by self-reactive T-cells, as autoreactive T-cells against 21-hydroxylase have been found present in AAD patients (107-109). Other possible mechanisms for development of AAD could be viral infections, genetic predisposition and use of check-point inhibitors (section 1.8) (92, 104) (Fig. 5). This illustrates the complexity of the disease mechanism.

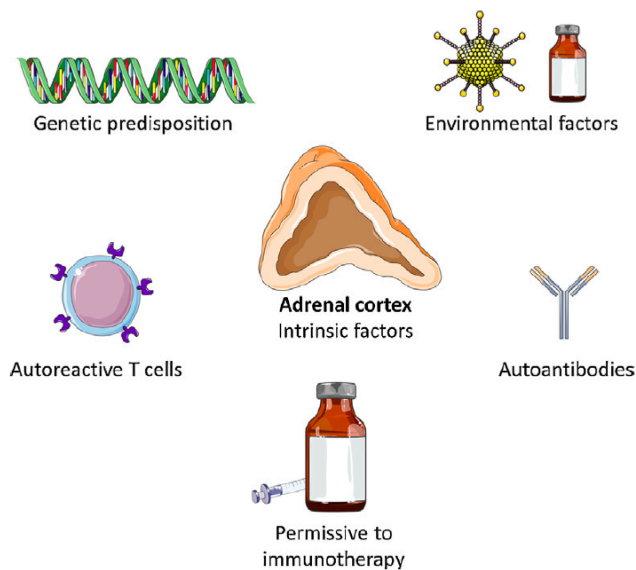

Figure 5: Factors that might contribute to the development of AAD in humans. *Environmental factors, such as a viral infection, or a person being genetically susceptible for autoimmunity are thought to be a part of AAD pathogenesis. As are the use of check-point inhibitors, autoantibodies and autoreactive T-cells. Figure from Hellesen et al., 2018 (104).*

Over time, patients will eventually start developing symptoms of disease, including skin hyperpigmentation and an increased hunger for salt, in addition to more diffuse features such as tiredness, nausea, low blood pressure and abdominal pain (105, 110). Furthermore, measured serum levels of cortisol are low, with an additional high level of adrenocorticotropic hormone (ACTH), in the morning (103, 110-112). Patients might also present with decreased levels of aldosterone, a hormone important in the body's mechanism working to maintain a normal salt-water balance (111, 112). If the disease is left untreated or the medication is insufficient, it may lead to the development of acute adrenal crisis, which in turn will lead to hospitalization and a need for treatment with intravenous hydrocortisone and saline (110, 112).

1.10. Regulatory T-cells and CTLA4 as targets for therapy in autoimmune Addison's disease

The fact that Tregs play an important role in maintaining self-tolerance and in the prevention of autoimmunity is now an established concept (38, 51, 113), but it is not yet known whether the frequency and/or function of Tregs is impaired in patients with AAD. What is known is that Tregs constitutively express CTLA4 (46) and that it plays a role in the development and progression of autoimmune diseases (94, 114). It has also been suggested that variants in the *CTLA4* gene could increase the risk for development of AAD (115). A 2015 study by Wolff *et al*. looked at the role of CTLA4 in AAD and found a variant that, in the European population, is associated with AAD, thus strengthening the fact that CTLA4 plays a part in the pathogenesis of the disease (116). A recent genome wide association study (GWAS) including patients with AAD from Norway and Sweden has further shown, with genome wide significance, that certain variants in the *CTLA4* gene predispose to AAD (Eriksson, Røyrvik and Aranda-Guillen *et al.*, submitted manuscript). All together these evidences make both Tregs and CTLA4 possible targets for therapy in AAD.

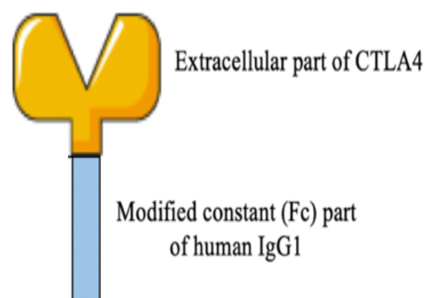

Figure 6: Structure of abatacept. *Abatacept is a fusion protein as it consists of the extracellular part of CTLA4 and a modified constant part of human IgG1.The figure contains elements from Servier Medical Art by Servier, 2020. Modified from Dubois et al., 2009 (118).*

There exists a drug on the marked today, abatacept, sold under the brand name Orencia (Bristol-Myers Squibb), that is a fusion protein made up of a modified constant part of human immunoglobulin 1 (IgG1) and the extracellular part of human CTLA4 (117, 118) (Fig. 6). It used in the treatment of rheumatoid arthritis, psoriatic arthritis and juvenile idiopathic arthritis, and works by interfering with the co-stimulatory signal that T-cells depend upon to become fully activated (117) (Fig. 7). Abatacept is available both as prefilled syringes and as a powder, where the latter is given to the patient as an infusion (117). Production of abatacept involves ovarian cells from Chinese hamsters and the use of recombinant DNA technology (117). Common side effects include upper airway infections and reactions at the site of infusion/injection (117). More serious infections, such as pneumonia, have also been reported, and the development of malignancy/cancer is another possible, but rare, side effect (117).

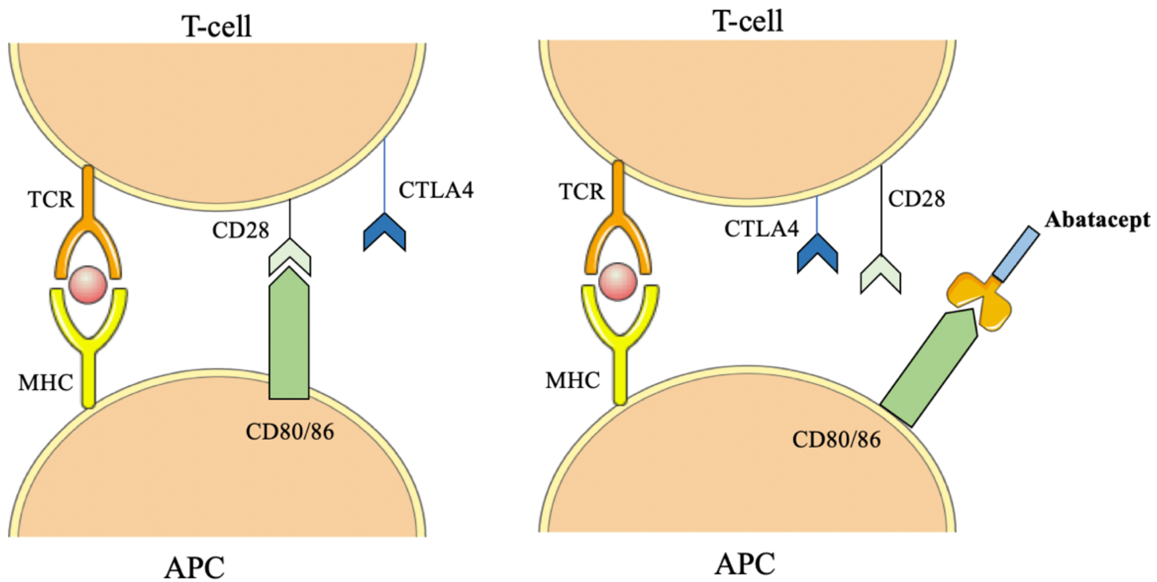

Figure 7: Abatacept mechanism of action. *Abatacept will block binding of CD28 to CD80/86 on the antigen presenting cell. The result is decreased production of cytokines and other inflammatory substances, due to inhibition of T-cell activation, proliferation and differentiation. The figure contains elements from Servier Medical Art by Servier, 2020. Modified from Dubois et al., 2009 (118).*

The mechanism of abatacept has been confirmed by Davis *et al.*, who have shown that the drug is a potent modulator of T-cell activation, as treated cells showed reduced proliferation and a decreased production of cytokines and interleukins, such as IL2 and IFN-γ (119). The effect of abatacept/CTLA4-Ig on Tregs in patients with rheumatoid arthritis have also been explored, but with conflicting results. One study indicated a lowered amount of Tregs, but with an increased regulatory/suppressive function, after treatment with abatacept (120). Another study interestingly reported an increased frequency of both T-cells and Tregs as a result of administration of CTLA4-Ig (abatacept) (121). Although the results of using abatacept as a modulator of Tregs is conflicting and mostly done in systemic autoimmune disorders, the use of abatacept has to date not been investigated in regard to AAD.

1.11. Current status

As elaborated on before, the immune depressor CTLA4, invariably expressed on Tregs (46), has been associated with AAD (115, 116) (Eriksson, Røyrvik and Aranda-Guillen *et al.*, submitted manuscript). CTLA4 is known to be involved in autoimmunity (94, 102, 114) and abatacept, a CTLA4-Ig fusion protein, is approved for use in the treatment of systemic autoimmune disorders, like autoimmune rheumatoid arthritis (117). Abatacept has also been tested in patients with recent-onset type 1 diabetes mellitus, indicating a delayed reduction in

the function of insulin producing cells in the pancreas, for a certain amount of time post-diagnosis (122, 123). Abatacept has, as far as we know, not yet been tried in patients with AAD or polyendocrine syndromes.

There exists, until now, only one treatment option for AAD patients; steroid replacement therapy (110, 111). Drugs are normally given as tablets, and it is important for patients to learn how to adjust the dosage, especially in stressful situations, like exercising or in the case of an infection (110). Studies have been conducted to test administering cortisone as an infusion through a pump, similar to the insulin pump used in the treatment of type 1 diabetes, but it has been difficult to obtain a precise mimic of the body's circadian cortisol production (124, 125). Due to the unphysiological treatment patients with AAD still suffer from numerous symptoms such as lower quality of life (126, 127) and a higher risk of death (128). Also, these patients will have to take medications and be taken care of by the specialised health care system for the rest of their lives (112). Therefore there is a great need to develop new treatment options, and preferably drugs that can redirect the autoimmune pathological reaction, and perhaps even reverse disease.

1.12. Hypothesis and aims

Our hypothesis is that CTLA4-Ig (here, further referred to as abatacept) could be used in the treatment of AAD by increasing the number of Tregs and suppress the autoimmune response by enhancing the inhibitory function of CTLA4. To examine this, we aimed to develop a method for drug screening and examine the effect of abatacept in an *in vitro* cell system.

Specific aims:

1. Develop an *in vitro* cell assay to screen the effect of drugs on T-cells and Tregs in AAD patients.

2. Examine the specific effect of abatacept on T-cells in patients with AAD by
    a. Use flow cytometry to examine cell expression of intra- and extracellular markers of T-cells and Tregs at the protein level.
    b. Use enzyme-linked immunosorbent assay (ELISA) to examine production and secretion of the cytokine IFN-γ in activated cells, treated or untreated with abatacept.

3. Examine gene expression in the T-cell signalling pathway in resting cells and in activated cells with or without abatacept by quantitative polymerase chain reaction (qPCR) at the RNA level.

2. Materials

The following tables show the different chemicals (table 1), equipment (table 2), instruments (table 3) and software (table 4) used in this project.

Table 1: Chemicals

Chemicals	Producer	Catalog number
2-Mercaptoethanol	Aldrich Chemistry	M6250-100ML
AB-serum	Sigma-Aldrich	H4522-100ML
Anti-CD3, V500, clone UCHT1	BD BioSciences	561416
Anti-CD4, Alexa Fluor 700, clone RPA-T4	BD BioSciences	557922
Anti-CD8, PerCP-Cy5.5, clone SK1	BD BioSciences	565310
Anti-CD25, PE-Cy7, clone 2A3	BD BioSciences	335824
Anti-CD31 (PECAM-1), BV785, clone L133	BD BioSciences	744757
Anti-CD39 (ENTPD-1), PE, clone eBioA1	Invitrogen by Thermo Fisher Scientific	12-0399-42
Anti-CD45RA , APC-H7, clone HI100	BD BioSciences	560674
Anti-CD152 (CTLA4), BV421, clone BNI3	Sirigen/BioLegend	369606
Anti-CD304 (neuropilin-1), BV650, clone U21-1283	BD BioSciences	743131
Anti-FoxP3, PECF594, clone 236A/E7	BD BioSciences	563955
Anti-Helios/IKZF2, APC, clone 22F6	BioLegend	137222
BD Pharm Lyse Lysing Buffer	BD Biosciences	555899
BioWhittaker RPMI 1640 without L-Glutamine	Lonza	BE12-167F
Bovine Serum Albumin (BSA)	Sigma Life Science	A7030-100G
DEPC-treated water	Ambion	AM9922
DMSO	Sigma-Aldrich	D2650
Ethanol 96%	Antibac	6000051
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F7524
Human BD Fc block	BD Biosciences	564220
Human CD152 [CTLA-4]:Fc	Chimerigen/Nordic BioSite	CHI-HF-220A4-C500
IL2	Thermo Fisher Scientific	PHC0023
L-Glutamine	Lonza	BE17-605F
MACS BSA Stock Solution	Miltenyi Biotec	130-091-376
Penicillin-Streptomycin Mixture	Lonza	17-602E
PBS (phosphate-buffered saline)	Sigma-Aldrich	D8537-500ML
RNase away	Molecular Bio Products	7002
RNaseZap	Thermo Fisher Scientific	AM9780, AM9782

RT^2 SYBR Green Rox qPCR Mastermix	Qiagen	330521
Stop Solution for TMB Substrate	BioLegend	423001
Surface disinfection 75%	Antibac	600521
TaqMan Gene Expression Assay (VIC). Gene symbol: B2M	Thermo Fisher Scientific	4448490
TaqMan Gene Expression Assay (FAM). Gene symbol: CTLA4	Thermo Fisher Scientific	4331182
TaqMan Gene Expression Assay (FAM). Gene symbol: FASLG	Thermo Fisher Scientific	4331182
TaqMan Gene Expression Assay (FAM). Gene symbol: ICOS	Thermo Fisher Scientific	4331182
TaqMan Gene Expression Assay (FAM). Gene symbol: JUN	Thermo Fisher Scientific	4331182
TaqMan Gene Expression Master Mix	Applied Biosystems/Thermo Fisher Scientific	4369016
Trypan Blue stain	Life Technologies Co	T10282
Tween 20	Sigma-Aldrich	P7949
Water Nuclease Free	VWR	7732-18-5

Table 2: Equipment and kits

Equipment	Producer	Catalog number
96-Well Immuno Plates	Thermo Fisher Scientific	6905TS
384 Well Multiply-PCR Plate skirted, natural	Sarstedt	72.1984.202
Agilent RNA 6000 Pico Kit	Agilent Technologies	5067-1513
Agilent RNA 6000 Pico Ladder	Agilent Technologies	5067-1535
Agilent RNA 6000 Pico Reagents	Agilent Technologies	5067-1514
ART Barrier Hinged Racked Pipette Tips - 1000 µL - 300 µL - 200 µL - 100 µL - 20 µL - 10 µL	Thermo Fisher Scientific	 2179-HR 2070-HR 2069-HR 2065-HR 2149P-HR 2139-HR
ART Barrier Reload Insert, Extended Length Pipette Tips - 1000 µL - 20 µL - 10 µL	Thermo Fisher Scientific	 2179-05-RI 2149E-05-RI 2140-05-RI
BD Vacutainer CPT	BD	362780/362753
CellTrace^{TM} CFSE Cell Proliferation Kit	Invitrogen	C34554
ClipTip Filtered Pipette Tips	Thermo Fisher Scientific	

- 300 µL		94420518
ClipTip Non-Filtered Pipette Tips - 300 µL	Thermo Fisher Scientific	 94410610
CoolCell FTS30	Biocision	BCS-170
Cryogenic Vials	Nalge Company	5000-1020 1.5 mL
Dead Cell Removal Kit	Miltenyi Biotech	130-090-101
eBioscience Foxp3/Transcription Factor Staining Buffer Set Kit	Invitrogen/Thermo Fisher Scientific	00-5523-00
ELISA MAX Deluxe Set Human IFN-γ	BioLegend	430104
F1-ClipTip Multichannel Pipettes - 30-300 µL	Thermo Fisher Scientific	 4661180N
Falcon tube - 15 mL - 50 mL	VWR	 525-0150 525-0156
Finnpipette F1 Multichannel Pipette 3-300 µL, 8 channels	Thermo Fisher Scientific	 4661030N
Finnpipette F1 Variable Volume Single-Channel Pipette - 0.2-2 µL - 0.5-5 µL - 1-10 µL - 2-20 µL - 10-100 µL - 20-200 µL - 100-1000 µL	Thermo Fisher Scientific	 4641020N 4641010N 4641030N 4641050N 4641070N 4641080N 4641100N
FLOWMI^{TM} Cell Strainers, 40 uM	SP Scienceware	136800040
Flow – tube	Corning Science	352063
Live/dead Fixable Yellow Dead Cell Stain Kit	Life Technologies	L34959
LS Columns	Miltenyi Biotec	130-042-401
MACSxpress Separator	Miltenyi Biotec	130-098-308
MACSxpress Whole Blood Treg Isolation Kit, human	Miltenyi Biotec	130-109-557
MicroAmp 8-Cap Strip	Applied Biosystems	N8010535
MicroAmp Optical Adhesive Film	Applied Biosystems	4311971
MicroAmp 8-Tube Strip (0.2 mL)	Applied Biosystems	N8010580
MidiMACS separator	Miltenyi Biotec	130-042-302
MS Columns	Miltenyi Biotec	130-042-201
Nitrile Medical Examination Gloves M	Abena	290498
Pasteur pipette	VWR	VWRI612-1683
PAXgene Blood RNA Kit	Qiagen	762174
PAXgene Blood RNA Tubes	Qiagen	762165

Pipetboy acu 2 Pipette Controller	Integra Biosciences	-
QIAshredder (250)	Qiagen	79656
RNase-free Elution Tubes	Thermo Fisher Scientific	AM12480
RNeasy Plus Micro Kit (50)	Qiagen	74034
RNA Pico Chips	Agilent Technologies	5067-1513
RT^2 First Strand Kit	Qiagen	330401
RT^2 Profiler^{TM} PCR Array Human T Cell Anergy & Immune Tolerance	Qiagen	330231
Safe-LockTubes 0.5 mL	Agilent	5065-9940
Safe-Lock Tubes 1.5 mL, yellow	Eppendorf AG	0030 120.159
Scepter Sensors 40 µm	Millipore Corporation	PHCC40050
Serological Pipet 25 mL	Falcon	P8250
Sterile Disposable Plastic Pipettes 10 mL	Sterilin	47510
SuperScript III First-Strand Synthesis System for RT-PCR	Invitrogen/Thermo Fisher Scientific	18080-051
VWR Reagent Reservoirs	VWR	89094-680
Zellkultur Testplatten 96U	TPP Techno Plastic Products AG	92697

Table 3: Instruments

Instrument	Producer	Catalog number
ABI Prism 7900 HT Sequence Detection System and SDS Enterprise Database	Applied Biosystems/Thermo Fisher Scientific	-
Agilent 2100 Bioanalyzer	Agilent Technologies	G2939BA
BD LSRFortessa	BD Biosciences	-
Centrifuge 5810	Eppendorf AG	5810000017
GeneAmp PCR System 9700	Applied Biosystems/Thermo Fisher Scientific	-
Heraeus FRESCO 21 Centrifuge	Thermo Fisher Scientific	75002555
Heraeus MULTIFUGE 3SR+ Centrifuge	Thermo Fisher Scientific	-
IKA VORTEX 1	IKA	0004047700
Incubating Mini Shaker	VWR	12620-942
Milli-Q IQ 7003/05/10/15 Water Purification System	Merck	-
MiniSpin centrifuge	Eppendorf	5452000018
NanoDrop ND-1000 UV-Vis Spectrophotometer	Thermo Fisher Scientific	-
Scepter Handheld Automated Cell Counter	Millipore Corporation	PHCC00000
SpectraMax Plus 384 Spectrophotometer	Molecular Devises LLC	-

Thermomixer compact	Eppendorf/Sigma-Aldrich	-
Veriti 96 well Thermal Cycler	Applied Biosystems/Thermo Fisher	4375786

Table 4: Software

Software	Producer
2100 Expert 2.6	Agilent Technologies
BD FACSDiva	BD Biosciences
FlowJo 10.4	FlowJo, LLC
GraphPad Prism 8	GraphPad Software
Microsoft Excel 2016	Microsoft
ND-1000 3.8	Thermo Fischer
SDS 2.3	Applied Biosystems
SoftMax Pro	Molecular Devices

3. Methods

3.1. Ethical considerations and the ROAS registry

The project has approval from the regional committees for medical and health research ethics (REK), with REK-numbers 2013/1504 and 2018/1417. All patients and healthy controls have provided informed written consent to donate blood to research. The patients are members of the registry for organ specific autoimmune diseases (ROAS), while healthy controls were recruited from blood donors at Haukeland University Hospital. The registry is a part of the section for endocrinology at Haukeland University Hospital (129) and has grown to be one of the world's largest through collaboration with hospitals and labs world-wide (130). ROAS was established in 1996 and is a national registry with information from 819 patients with AAD, 313 patients with hypoparathyroidism and 43 patients with APS-1 (data from 2018) (131) and also include a biobank with serum and blood samples. New patient samples are obtained during the patients' yearly follow-up with a specialist in endocrinology.

3.2. Experimental pipeline

All the methods used in this project are described in the following sections. The flow chart below (Fig. 8) gives an overview of the experimental pipeline, which is divided into two parts. Part one (Fig. 8 top) involves examining gene expression in the T-cell signalling pathway in non-activated cells by quantitative polymerase chain reaction (qPCR). The second part (Fig. 8 bottom) uses cell culture, enzyme-linked immunosorbent assay (ELISA) and flow cytometry to examine the effect of abatacept on T-cells, and qPCR to look at differences in gene expression in the T-cell signalling pathway in cells treated or not treated with abatacept. All samples used in part two (Fig. 8 bottom) are divided into the following groups

Patient no drug
Patient drug
Control no drug
Control drug

where "no drug" means no addition of abatacept, while "drug" means that the sample is treated with abatacept. This applies to all results, with the exception of qPCR on non-activated cells (section 4.3).

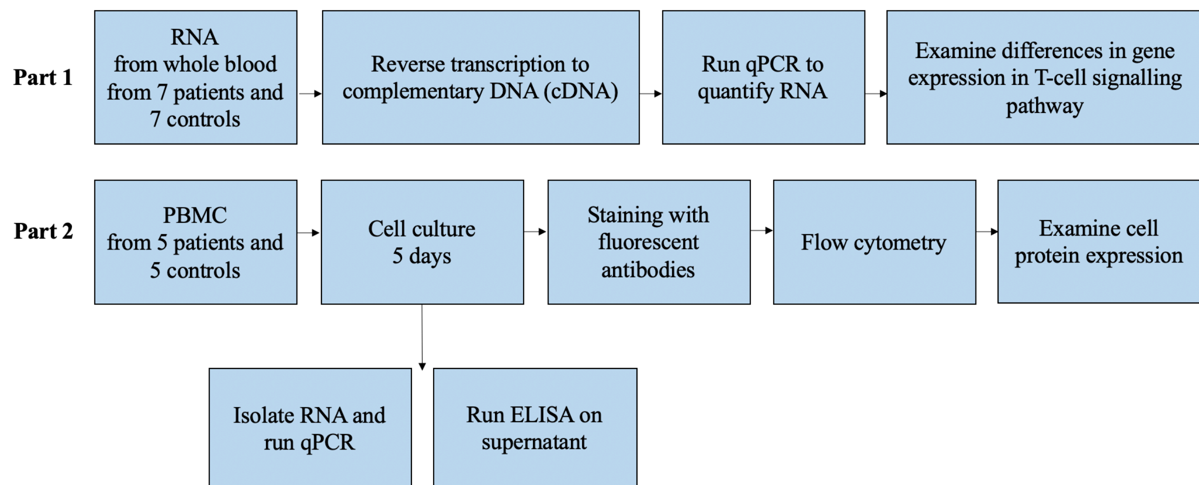

Figure 8: Experimental pipeline. *The upper part of the figure describes part 1 which involves examining differences in gene expression between patients with AAD and healthy controls. The bottom part of the figure describes part 2, which involves examining cell protein expression and the effect of abatacept on T-cells in patients with AAD.*

3.3. Patients and controls

AAD patients 1-5 (3 males, 2 females, age range 25-61, mean age 41.6) and healthy controls 1-5 (3 males, 2 females, age range 28-54, mean age 40.0) were included in the cell culture and subsequent analysis by flow cytometry, ELISA and qPCR. RNA from APS-1 patients 6-11 (3 male, 3 female, age range 22-50, mean age 36.17 years, all had Addison's disease), AAD patient 12 (female, age unknown) and healthy controls 6-12 (3 male, 4 female, age unknown) were used in the qPCR experiment from resting cells. Additional data on patients 1-12 and controls 1-5 can be found in Appendix I. For controls 6-12 the only available data was that they were age- and sex-matched with patients 6-12.

3.4. Choice of methods

Flow cytometry is one of the central techniques in this study. Here, lymphocytes derived by isolation of PBMC are stained with fluorochrome-conjugated antibodies, specific for the surface- and intracellular T-cell and Treg markers of interest, to examine cell protein expression. When the fluorochromes passes through the laser beam in the flow cytometer, it will absorb light at one wavelength, before being excited to a higher level of energy (132). The fluorochromes will almost immediately fall back to the ground state, resulting in fluorescence and the emittance of light at a different wavelength, creating a fluorescent signal measured by the flow cytometer (132). Filters in the instrument will take up the emitted light, thus permitting

distinct wavelengths to pass through (132). Flow cytometry also enables us to investigate how the cells scatter light. There are two possible scattering directions, forward (FCS) and side (SSC); FCS will give information about cell size (increasing with larger cells), while SSC will give information about cell granularity and complexity (increasing with higher complexity) (132).

qPCR was used to examine expression of selected genes in the T-cell signalling pathway in non-activated cells and in activated cells, treated or untreated with abatacept. Being a quantitative method, qPCR can determine the amount of cDNA present, which again directly represent the mRNA level in the specimen, and provides opportunities to further normalize the data and calculate fold change values by the $\Delta\Delta C_t$-method (133-135), which is described in section 3.7. This project involves the use of two different chemistry bases for qPCR; TaqMan and SYBR Green. With TaqMan it is necessary to have a probe with a fluorescent reporter dye on the 3' end and a quencher attached to the 5' end, in addition to a pair of primers (133, 135). During the DNA amplification process, cleavage of the probe takes place as a result of the 5'-nuclease activity of the TaqMan polymerase enzyme, which leads to the reporter being separated from the quencher, followed by the generation of a fluorescent signal, detected by the qPCR-instrument (133, 135, 136). SYBR Green requires a fluorescent dye that has the ability to bind the double stranded DNA qPCR product, giving rise to a fluorescent signal detected by the instrument (133, 135).

A sandwich ELISA was here applied to detect secretion of INF-γ by activated cells treated or not treated with abatacept. The principle is to first coat a plate with an antibody specific for the antigen/antibody of interest, in this case a mouse antibody specific for human INF-γ (7, 137). The addition of standard solutions and, in this case, patient/control samples will enforce binding of INF-γ to the immobilized capture antibody, and an antibody-antigen-antibody "sandwich" is generated by the further addition of INF-γ detection antibody (137). Wells containing cells producing INF-γ will change colour to blue when avidin-horseradish peroxidase (HRP) and 3,3',5,5'-tetramethylbenzidine (TMB) substrate solutions are added (137). The intensity of the blue colour is proportional to the concentration of INF-γ present in the unknown samples, and the addition of stop solution will turn the colour of the solutions to yellow (137), before being read by the SpectraMax Plus 384 spectrophotometer, at wavelength 450 nm.

3.5. Isolation of PBMC

PBMC was isolated from five healthy controls and five AAD patients. In short, a blood sample in a BD vacutainer cell preparation tube (CPT), was centrifuged at 1800 G at 20°C for 15 minutes. The PBMC layer was pipetted out directly and transferred to a 15 mL Falcon tube and washed with phosphate-buffered saline (PBS). Next followed centrifugation at 300 G at 20°C for 15 minutes; the supernatant was removed. The pellet was resuspended in PBS and the cells were counted by Scepter handheld automated cell counter. The cells were then centrifuged at 300 G at 20°C for 10 minutes, and the pellet was resuspended in 250 µL AB human serum. A solution containing human AB serum and dimethyl sulfoxide (DMSO) was made in a 1:4 ratio, and 250 µL was added to the resuspended cells. The cells were transferred to a cryo vial, and stored in room temperature for 15 minutes, before being transferred to a cell freezing container and stored at -80°C for two days and further at -150°C until use.

3.6. Isolation of RNA from whole blood and profiling of gene expression related to immune tolerance and T-cell anergy

Blood from six APS-1 patients (all with Addison's disease), one AAD patient and seven healthy controls was drawn from the donor directly into PAXgene Blood RNA Tubes and RNA was obtained by using the PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's protocol. The RNA was further transcribed to complementary DNA (cDNA) by the Qiagen RT^2 First Strand Kit. The cDNA was next prepared for qPCR by the Qiagen RT^2 Profiler PCR Array Human T Cell Anergy & Immune Tolerance Kit, both according to the manufacturer's protocol. In short, cDNA was synthesised from RNA by making a genomic DNA elimination mix, followed by incubation at 42°C for one minute and placement on ice. Next, the reverse transcriptase mix was prepared and added to the cDNA elimination mix, followed by incubation at 42°C for 15 minutes, before the reaction was stopped with a new incubation at 95°C for five minutes. The RT^2 SYBR Green ROX qPCR Mastermix was prepared and added to the cDNA, before being transferred to a 384-well plate in a 4 x 96 PCR array. Samples were run for 40 cycles on the ABI Prism 7900 HT (table 5), using the SDS 2.3 software.

Table 5: Program used to run qPCR on ABI Prism 7900HT.

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

The plate used includes 84 different genes in the immune tolerance and anergy pathway, in addition to six housekeeping genes, one genomic DNA control, three empty wells and six RT/PCR controls in a 4 x 96 array format (138). All genes involved in the analysis can be found in appendix II. The fold change was calculated by the use of the $\Delta\Delta C_t$-method in Excel. When using the $\Delta\Delta C_t$-method, the C_t-values obtained from the qPCR-instrument were first normalized in reference to an endogenous control, usually a housekeeping gene, to obtain a ΔC_t-value (134). The data were further normalized relative to a calibrator, which gives a $\Delta\Delta C_t$-value, and $2^{-\Delta\Delta C_t}$ further gives the fold change (134). In this case, the mean of housekeeping genes was used as reference, while the mean of healthy controls was used as calibrators.

3.7. Cell culture and flow cytometry

T-cells from five AAD patients and five healthy controls were activated and prepared for flow cytometry according to the following protocol.

Coating with anti-CD3 antibody

Anti-CD3 antibody was diluted to a concentration of 1 µg/mL in sterile PBS and 50 µL was added to the wells of a 96-well plate. The plate was incubated at 37°C and 5% CO_2 for two hours or at 4°C overnight. The wells were washed with 180 µL PBS three times, before 100 µL cell suspension was added to the wells.

Preparation of cell medium and carboxy fluorescein diacetate succinimidyl ester (CFSE) solution

Cell medium was supplemented with 50 mL fetal bovine serum (FBS), 20000 μg/mL penicillin-streptomycin and 200 mM L-glutamine. CFSE solution was prepared by using the Invitrogen CFSE Cell Proliferation Kit, to a concentration of 10 µM.

Removal of dead cells

The cells were added to 10 mL warm medium and centrifuged at 300 G for 10 minutes, before the Miltenyi Biotech dead cell removal kit was used according to the manufacturer's protocol. The cells were counted using a Scepter handheld automated cell counter and further washed with medium at 300 G for 10 minutes.

Activation and functional characterization of the cells

Cells were resuspended in 1 mL warm PBS, 1 mL PBS-CFSE solution was added and the solution was incubated at 37°C for 10 minutes. Next, 2 mL cold PBS was added, followed by a five minute incubation on ice. Medium was added to 15 mL, before centrifugation at 300 G for 10 minutes. The pellet was then resuspended in warm medium to two million cells. IL2 was added to the cell suspension in a 2 μL/mL concentration.

Human CTLA4-Ig (ordered from Chimerigen/Nordic BioSite and here referred to as abatacept) was reconstituted to 100 μg/mL in 1 mL sterile PBS and stored in aliquots of 250 μL at -20°C until use. A dilution series was made to determine the concentration of abatacept to be used in further analysis. Abatacept was prepared in spanning concentrations between 0.65 and 20 μg/mL diluted in medium. Further, 100 μL cell suspension was transferred to the coated plate, together with 100 μL abatacept in given concentrations, giving a total volume of 200 μL in each well. In minimum two samples, for both patients and controls, the drug was replaced by 100 μL medium. Finally, the plate was incubated at 37°C and 5% CO_2 for five days. After three days the cells were split by transferring 100 μL from each well to a new plate and added 100 μL medium. After five days the supernatant from each well was harvested and collected in Eppendorf tubes, two wells were saved for RNA isolation, while the remaining cells were prepared for flow cytometry.

Preparation for flow cytometry

Lymphocytes derived by isolation of PBMC and cultured for five days were stained with fluorochrome-conjugated antibodies specific for the surface- and intracellular markers of interest (table 6) and analysed by flow cytometry. The panel has been validated by Heimli in a 2018 master thesis project (139) and consists of surface- and intracellular markers for different subpopulations of T-cells, with specific emphasis on Tregs, based on work by Santegoets *et al.* (65).

Table 6: Panel for flow cytometry including targets, dilution factors, excitation and filter for emittance.

Target	Fluoro-chrome	Dilution factor	Excitation [nm]	Filter for emittance
CD3	V500	1:20	407	670/30 band pass
CD4	Alexa Fluor 700	1:160	640	730/45 band pass
CD8	PerCP-Cy5.5	1:20	488	695/40 band pass
CD25/IL-2RA	PE-Cy7	1:40	561	780/60 band pass
CD45RA	APC-H7	1:80	640	780/60 band pass
CD152/CTLA4	BV421	1:20	407	450/50 band pass
CD39/ENTPD-1	PE	1:500	561	582/15 band pass
CD31/PECAM-1	BV785	1:160	407	780/60 band pass
CD304/Neuropilin-1	BV650	1:80	407	670/30 band pass
FoxP3	PE-CF594	1:10	561	610/20 band pass
Helios/IKZF2	APC	1:40	640	670/14 band pass
Dead cell stain	Q-dot585	1:1000	407	585/42 band pass
CFSE	FITC	-	488	530/30 band pass

The cell suspensions were prepared for flow cytometry, according to the following protocol; The cells were transferred from the plate into flow tubes before centrifugation at 350 G and 4°C for 10 minutes. The supernatant was removed and stored at -80°C, until ELISA analysis, and the remaining pellet was resuspended in 1 mL PBS. Dead cell stain was dissolved in 50 µL DMSO and 1 µL was transferred to the samples, before incubation in the dark at room temperature for 20 minutes. Next followed washing with 1 mL PBS with 0.5% BSA and centrifugation at 350 G and 4°C for 10 minutes. Supernatant was then removed, and the pellet resuspended in the remaining buffer. Further, 2 µL 0.5 mg/mL Fc-block was added, followed by incubation in the dark at room temperature for 20 minutes, before the cells were washed as previously described.

A mastermix of the cell surface markers was made using anti- CD3, CD4, CD8, CD25, CD45RA, CD152, CD39, CD31 and CD304, and added to the samples, and incubated in the

dark at 4°C for 20 minutes, followed by a washing step. Anti-human FoxP3 staining kit for intracellular staining (Invitrogen) was used for fixation and permeabilization and 1 mL fixation/permeabilization buffer was added before incubation overnight at 4°C. The permeabilization process was completed by washing with 2 mL 1X permeabilization buffer and centrifugation at 500 G and 20°C for five minutes. The cells were then resuspended in the remaining buffer. FOXP3 and Helios were added according to the dilution factors in table 7. The samples were incubated at 4°C for one hour. Next followed washing with 2 mL 1X permeabilization buffer and centrifugation at 500 G and 20°C for five minutes. The cells were then resuspended in the remaining buffer and the volume was increased by adding 250 μL 1X permeabilization buffer before analysation on the BD LSRFortessa flow cytometer. Further data-analysis was done using the FlowJo 10.4 software.

3.8. Isolation of RNA from activated cells and qPCR on activated cells, treated or not treated with abatacept

To isolate RNA from activated cells, treated or untreated with abatacept, from five AAD patients and five healthy controls, the RNeasy Plus Micro Kit (Qiagen) was used following the manufacturer's protocol. First, cells from PBMC were resuspended in RLT buffer and run through a QIAshredder spin column, before being transferred to a genomic DNA elimination column. One volume 70% ethanol was added to the eluted fraction, followed by transfer to a RNeasy MinElute spin column. In the following steps, the column was washed with 700 μL RW1 buffer, 500 μL RPE buffer and 500 μL 80 % ethanol. To elute the RNA, 14 μL DEPC-water was added to the column. The last step included a double elution, where the water was run twice through the column. The concentration of RNA in each sample was measured on a NanoDrop ND-1000 spectrophotometer. According to the manufacturer's protocol, the Agilent RNA 6000 Pico Kit and an Agilent 2100 Bioanalyzer was used to examine the quality of the isolated RNA. The 2100 Expert Software was used for analysis and provided RNA integrity numbers (RIN). The samples were stored at -80°C until use.

Cells from five AAD patients and five controls activated with IL2, with or without the addition of abatacept, and grown in culture for five days were used for RNA extraction, as described above, and subsequent cDNA synthesis by the Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR. Genes were chosen based on results from the qPCR on non-activated cells from patients suffering from APS-1 (section 3.7). For each sample a mastermix for first-strand

synthesis containing 0.35 µL random hexamers, 0.35 µL oligo dT20 primer, 0.35 µL 10 mM dNTPs and 0.5 µL DEPC water was made and 1.5 µL was added to PCR-tubes. Next, 5 µL RNA was then added to the tubes and mixed by pipetting. The samples were incubated on the GeneAmp PCR System 9700 at 65°C for five minutes. cDNA was synthesized by making a mastermix containing 1 µL 10X RT buffer, 2 µL 25 mM $MgCl_2$, 1 µL 0.1 M DTT, 0.5 µL RNase out, 0.5 µL 200 U/µL SuperScript III RT and 1.5 µL DEPC water, and 6.5 µL was added to each sample, followed by incubation on the GeneAmp PCR System 9700 (table 7).

Table 7: Program run on GeneAmp PCR system 9700 to synthesize cDNA.

Stage	Temperature	Time
1	25°C	5 minutes
2	50°C	60 minutes
3	55°C	15 minutes
4	70°C	15 minutes
5	4°C	Storage

Next, the cDNA was diluted 50:50 with milli-Q water. A mastermix was made for each gene of interest, containing 1.65 µL 20X TaqMan probe/primer mix (*CTLA4*, Fas Ligand Gene (*FASLG)*, inducible T-cell co-stimulator (*ICOS*), *JUN*), 1.65 µL 20X housekeeping gene (*B2M*), 16.5 µL 2X TaqMan Gene Expression Mastermix and 9.35 µL DEPC water. Further, 28 µL of the mastermix was added to a 96-well plate and five µL cDNA was added to each well on the plate and mixed by pipetting. Each well on the 96-well plate was pipetted in triplicates, adding 10 µL sample solution, to wells of a 384-well plate. The plate was centrifuged at 1000 rpm for two minutes and 40 cycles were run on the ABI Prism 7900HT, according to the same program as shown in table 5.

The SDS 2.3 software provided C_t -values and further analysis was done in Excel by using the $\Delta\Delta C_t$- method, as described in section 3.7. Normalized gene expression was calculated by $2^{-\Delta C_t}$ (134). The housekeeping gene *B2M* was used as endogenous control, while the non-treated sample in each pair (patient no drug/patient drug, control no drug/control drug) was used as the calibrator. For the no drug samples $\Delta\Delta C_t$ equals zero and the fold change becomes one, and the fold change values for the drug samples will indicate an up- or downregulation in gene expression in reference to the no drug samples for both patients and controls (134).

3.9. ELISA

Supernatant from cells grown in culture were saved and used to run ELISA to detect INF-γ by the ELISA MAX Deluxe Set Human IFN-γ (BioLegend), according to the manufacturers protocol. A 96-well plate was coated with capture antibody solution and incubated at 4°C overnight. Next, the plate was washed four times with 300 μL wash buffer, which counts for all the following wash steps. Further, 1X assay diluent A was added to the wells before incubation on a plate shaker at 500 rpm for one hour. This was followed by a new wash step. Samples were diluted 1:25 and 1:100, and standards and samples were added to the wells in triplicates. The plate was next incubated with shaking at 500 rpm for two hours, before washing. Detection antibody was added to the wells, followed by incubation on the plate shaker at 500 rpm for one hour. The plate was washed, before Avidin-HRP solution was added. The plate was then incubated with shaking at 500 rpm for 30 minutes. This was followed by a five round wash step. Each wash was soaked on the plate for 30 seconds, before TBM substrate solution was added. This was followed by incubation in the dark for 20 minutes, before the addition of stop solution. The absorbance was measured at 450 nm by using the SpectraMax Plus 384 Spectrophotometer. Standard curve and INF-γ concentrations in each sample were acquired by the use of the SoftMax Pro software. The software also provided adjusted concentration values, taking the dilutions into consideration. The sensitivity of the ELISA kit was 4 pg/mL.

3.10. Statistical analysis

For the qPCR on non-activated cells (section 3.7), statistical analysis was done in a “default” spreadsheet (Excel) made by the manufacturer, using multiple t-tests. Statistical analysis for the remaining experiments was done using GraphPad Prism 8. When examining differences within the patient no drug/drug population and within the control no drug/drug population a paired parametric t-test was used. In order to compare patient and control samples, treated or not with abatacept, an unpaired non-parametric t-test was performed (Mann-Whitney test). For all statistical analyses, unless otherwise described in the text, a significant result was characterized by a p-value less than 0.05. The mean is represented in the plots even though a non-parametric approach has been chosen in some of the statistical calculations. When examining differences between patient and control groups, data are presented as mean percent point ± standard deviation and mean proliferation index ± standard deviation for the flow cytometry data, and mean concentration [pg/mL] ± standard deviation for the ELISA data.

4. Results

4.1. Isolation of PBMC

From isolation of PBMC a concentration of (0.500 – 9) x 10^5 cells/mL was obtained from each subject. Five age- and sex-matched controls were selected for use in cell culture, flow cytometry, ELISA and qPCR. Number of cells obtained in each sample used in isolation of PBMC can be found in Appendix III.

4.2. Isolation of RNA from activated cells treated or not treated with abatacept

RNA from five AAD patients and five age- and sex-matched healthy controls was isolated from activated cells, treated or untreated with abatacept, and grown in culture for five days. We obtained RNA concentrations ranging from 0.85 to 61.83 ng/mL. In general, the obtained concentrations of RNA from each sample were low. An example of an output from the Agilent 2100 Bioanalyzer is shown in Fig. 9. This sample is from control 5 with and without treatment. Two clear bands, representing 18S and 28S RNA, were obtained and observed on the gel (Fig. 9 (a)). The nucleotide number versus fluorescence graph gave two well-defined peaks, representing the 18S and 28S RNA, respectively (Fig. 9 (b)). RIN values of 9.10 (control 5 no drug) and 9.20 (control 5 drug) was here considered indicative of high-quality RNA (140). The isolated RNA was further used in qPCR analysis of activated cells, despite low concentrations and/or poor quality. Concentration- and RIN-values for patients and controls 1-5 can be found in Appendix IV. Due to low sample volume, it was not possible to obtain RIN-values for all samples (Appendix IV).

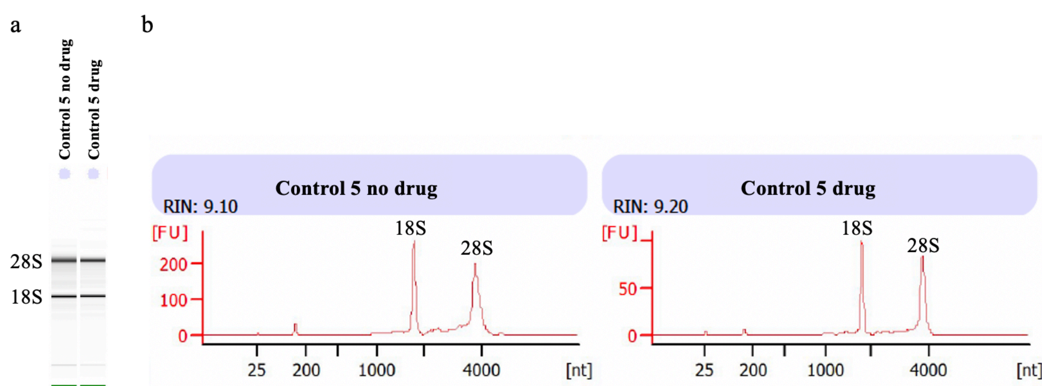

Figure 9: RNA quality result from the Agilent 2100 Bioanalyzer, here exemplified by samples from control 5. *(a) The gel picture showed two easily distinguishable bands, representing 18S and 28S RNA, respectively. (b) The nucleotide number versus fluorescence graph gave two well-defined peaks. The first one appearing represents 18S RNA, while the second represents 28S RNA. RNA integrity numbers of 9.10 (control no drug) and 9.20 (control drug) indicated RNA of good quality. All RNA samples were used in the qPCR experiment in activated cells, treated or not treated with abatacept, despite low concentration and/or poor quality.*

4.3. RNA expression profiling of genes related to immune tolerance and T cell anergy

In order to identify genes within the T-cell tolerance pathway, including *CTLA4*, which possibly differed between patients and controls, we utilized a commercial qPCR assay including 84 relevant genes. RNA was obtained from the ROAS registry, all with RIN values indicating high quality RNA (not shown), from seven AAD patients and seven age- and sex-matched controls were analysed for the expression of 84 genes. Genes with deviant expression between patients and controls was then further analysed in activated cells, treated or not, with a CTLA4 agonist (abatacept) in downstream experiments.

Results from the analysis of RT2 real time profiling data are shown in Fig. 10. Of the 84 genes, 52 amplified in all 14 samples (seven patients and seven controls), while 31 genes amplified in between 1 and 13 samples, with varying numbers of patients and controls involved. One gene did not give output for all seven control samples, only for one patient sample (*IL17A*), indicating very low expression. All wells passed the control steps, except for sample 1 in the control group, where there was a question about the efficacy of the reverse transcriptase process, but the sample was still included. Eleven genes are presented (Fig. 10) and were selected based on either a fold change value between patient and control larger than 3 and/or a p-value less than 0.05. Data for the remaining genes can be found in appendix II.

The instrument was set to run 40 cycles, and products appearing after this were considered undetermined due to low expression. These values were removed from the dataset and not taken into consideration. This leads to a lowered number of replicates, meaning that the number of patients and controls did not always match. The "default" spread-sheet used to analyse the data (from the manufacturer) had a cut-off value set as 35, this was changed to 40 by us, thus taking into consideration the values between 35 and 40. If there were less than three replicates from each group, statistical analysis was not done, but the fold change was still used as an indicator for differences. Taking ICOS as an example, a positive upregulation of 26.91 was observed (Fig. 10), but there was no obtained p-value. This was because there were only two controls left after removing undetermined and low-expression results. The fold change values (Fig. 10) reflects a positive or negative regulation compared to the patient group. A positive value indicated a higher expression in patient versus control, while a negative value indicated lower expression in patients versus controls. To correct for multiple testing in this experiment (since we were testing 84 genes in the same setup) the threshold for a significant result was decreased to 0.01. This implied that the change seen between patient and control for *FASLG* (Fig. 10) was

not considered statistically significant.

Six of the genes presented (*LTA, JAK3, IL13, IL10, IFNG, ICOS)* (Fig. 10) had an overall high threshold value, meaning that the expression was low for both patient and control samples, and the p-value was greater than 0.01, i.e. there was no statistical differences between the groups. In general, the expression of genes in both patients and controls were low, as many signals appeared late in the qPCR setup (high Ct value) (not shown).

We chose four genes on the basis of these qPCR results in resting cells to investigate mechanisms in response to abatacept in downstream applications for activated cells: *FASLG* (because it was close to obtaining significant adjusted p-value), *ICOS* and *JUN* (with relatively large fold change values between the groups) and *CTLA4* (because of its relevance for abatacept).

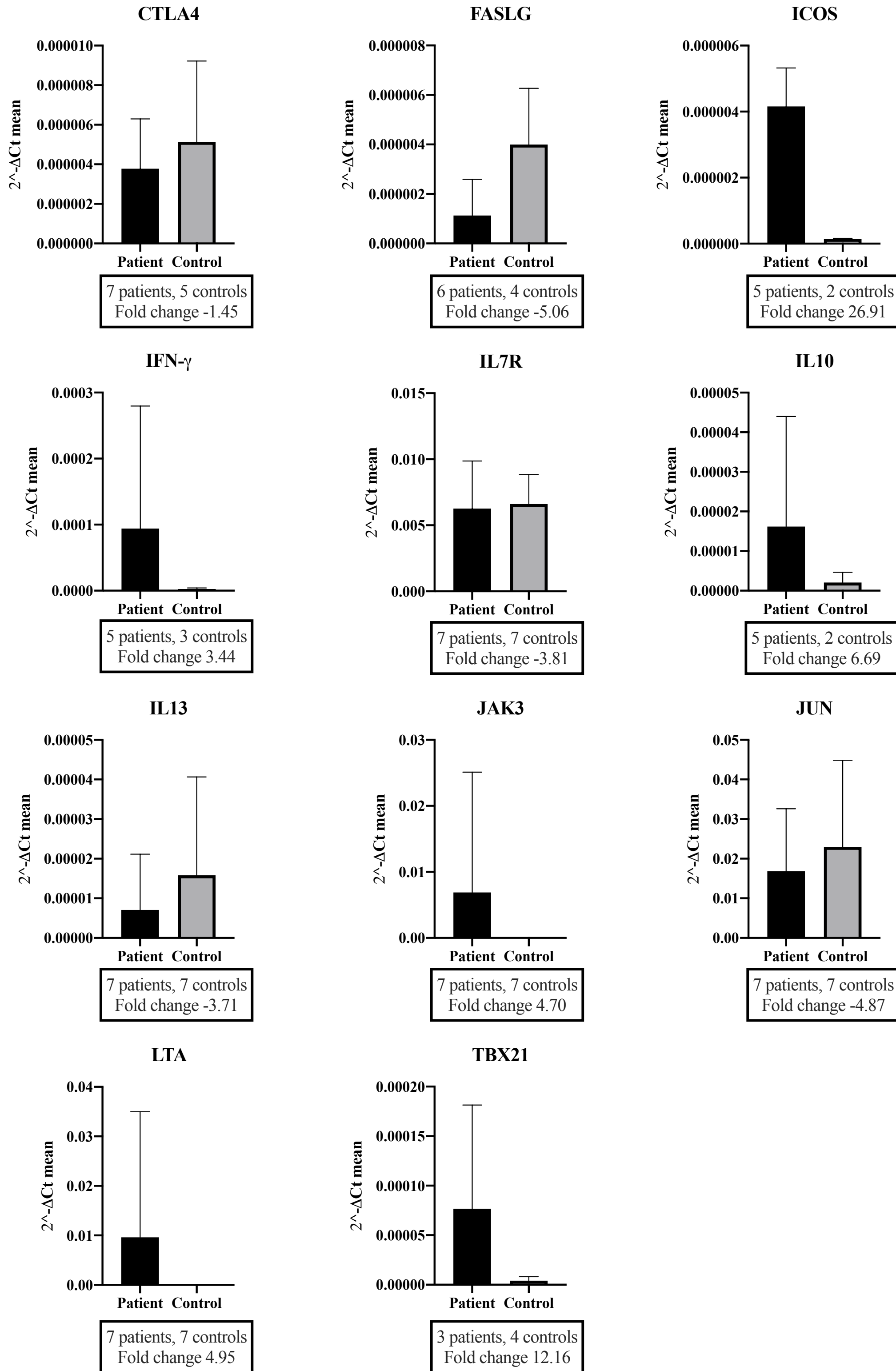

Figure 10: Results from RT2 real time profiling for 11 of the 84 genes. *The number of matched patients and controls for each gene are presented under the graphs. Fold change values are presented for all genes. A negative value means that the expression was higher in the control group, while a positive value means that the expression was higher in the patient group. The box represents the mean fold change value, while the red line indicates standard deviation. Statistical analyses was performed in a spreadsheet provided by Qiagen, using multiple t-tests.*

4.4. qPCR on activated cells, treated or untreated with abatacept

The isolated RNA samples from activated cells, with or without abatacept, from five AAD patients and five healthy controls were further investigated for gene expression of selected genes. A TaqMan qPCR assay was performed including four genes (*CTLA4, FASLG, ICOS, JUN*) that were chosen based on results from the qPCR done on non-activated cells from APS-1 patients, an AAD patient and healthy controls (Fig. 10, section 4.3). The qPCR-instrument was set to run 40 cycles and signals appearing after these cycles were excluded due to very low or non-existing expression. For control 3, in the analysis of *JUN*, no Ct-values were obtained, and the sample was thus removed. Therefore, five patients and four controls were a part of the *JUN* analysis. When comparing normalized gene expression ($2^{-\Delta C_t}$) before and after treatment with abatacept in patients and controls samples, we found no significant differences for the four candidate genes within the two groups (Fig. 11). Indeed, the expression of the selected genes were very low, appeared late in the qPCR cycle (not shown) and the results fluctuated greatly within the groups and between patients and controls (Fig. 11).

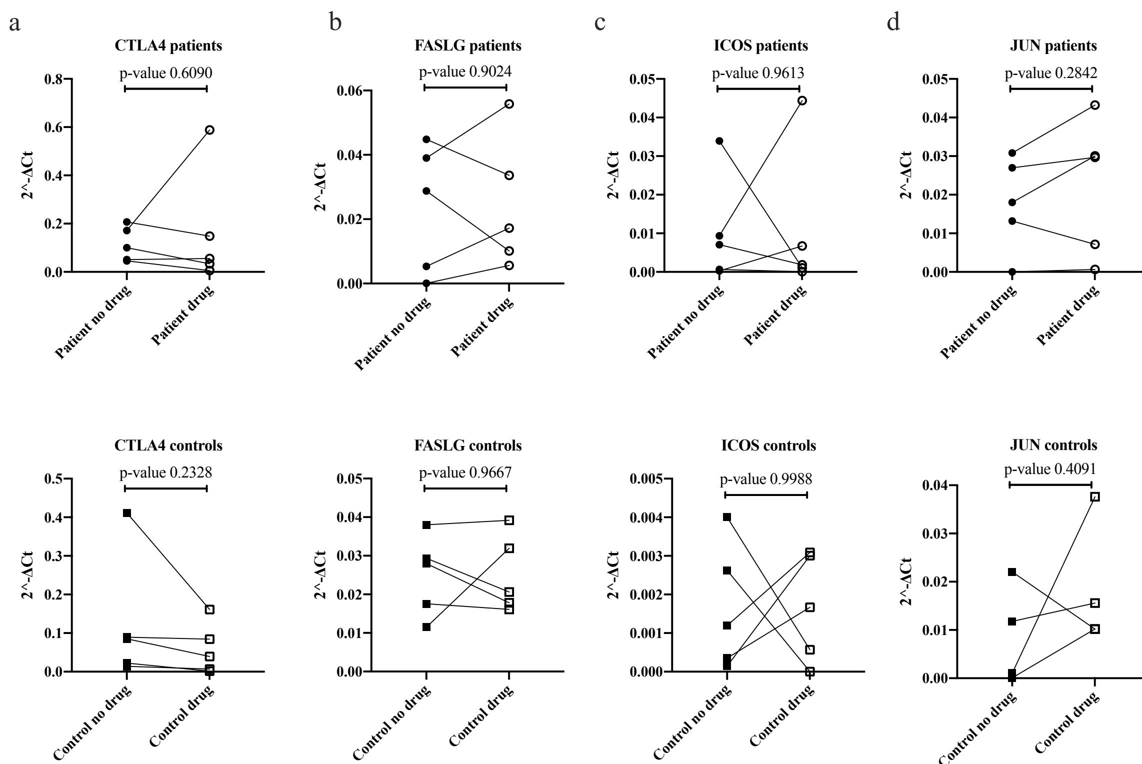

Figure 11: qPCR on activated cells, treated or not, with abatacept; normalized gene expression. *(a) CTLA4 patients and controls, (b) FASLG patients and controls, (c) ICOS patients and controls and (d) JUN patients and controls. Normalized gene expression, given as* $2^{-\Delta C_t}$ *makes up the y-axis, and gives the opportunity to compare expression before and after treatment with abatacept. Parametric paired t-tests did not give significant differences between patients no drug/drug and controls no drug/drug. There were no clear trends when looking at the results, as gene expression both increase and decrease when comparing the no drug and drug samples for individual subjects. The overall gene expression for all four genes, for both patients and controls, were low.*

Fold change values were calculated as described previously (section 3.7) and presented in Fig. 12. Since the no drug samples for both patients and controls were used as calibrators (section 3.9) these fold change values equals one, indicated by a horizontal line (Fig. 12). Fold change values were thus plotted for the patient drug and control drug groups and provided an indication of up- or downregulation for the samples treated with abatacept in relation to the samples with fold change values of one (not treated with abatacept). For *CTLA4*, samples from patient 1, 4 and 5 (treated with abatacept), showed a slight downregulation, while patient 2 had a higher expression (Fig. 12). Patient 3 did not show signs of up- or downregulation (Fig. 12). The control samples were all under the horizontal line for *CTLA4*, which could be an indication of a lower expression in the samples treated with abatacept, compared to the non-treated samples. Statistical analysis looking at differences between patient drug and control drug gave no significant differences. All genes in the patient population contained a sample with higher expression than the remaining samples, and for *FASLG, ICOS* and *JUN* this was the same sample, patient 5.

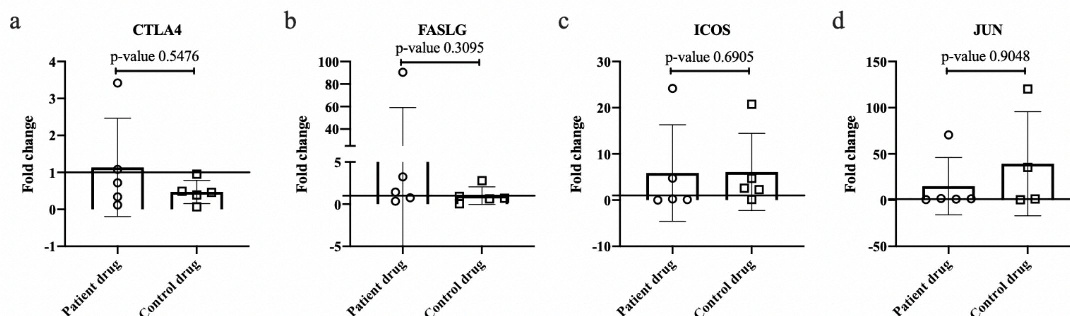

Figure 12: qPCR on activated cells treated abatacept; fold change values for patient and control samples treated with abatacept. *(a) CTLA4, (b) FASLG, (c) ICOS and (d) JUN. Fold change values for the no drug samples equal one, as they were used as calibrators, which is marked by the horizontal line. Statistical analyses (non-parametric unpaired t-test) performed between patients and controls treated with abatacept did not give any significant differences. These results indicate no change in gene expression after treatment with abatacept for neither patients nor controls, and that abatacept does not affect the expression of these genes. The boxes represents the mean, while the error bars show the standard deviation.*

4.5. Cell culture and flow cytometry

The effect of abatacept on T-cells was examined in five patients with AAD and five age- and sex-matched healthy controls by cell culture and flow cytometry. Based on preliminary titration data, an abatacept concentration of 20 μg/mL was chosen for use in the cell based assay. After being activated in cell culture for five days, the cells were analysed by flow cytometry, which was used to analyse at cell protein expression.

4.5.1. Gating strategy

An example of the gating strategies used is shown in figures 13-15, all showing control 1 with no drug. The gating strategy was the same for all remaining samples. First, gating was set for all cells present in the given sample, using an FSC versus SSC plot. Next, it was gated for single cells, using an FSC-A versus FSC-H plot. From single cells the gating was set to live cells. To make sure that we were only looking at T-cells, it was gated for $CD3^+$ cells and further for $CD4^+$ and $CD25^+$ $FOXP3^+$ cells (Fig. 13).

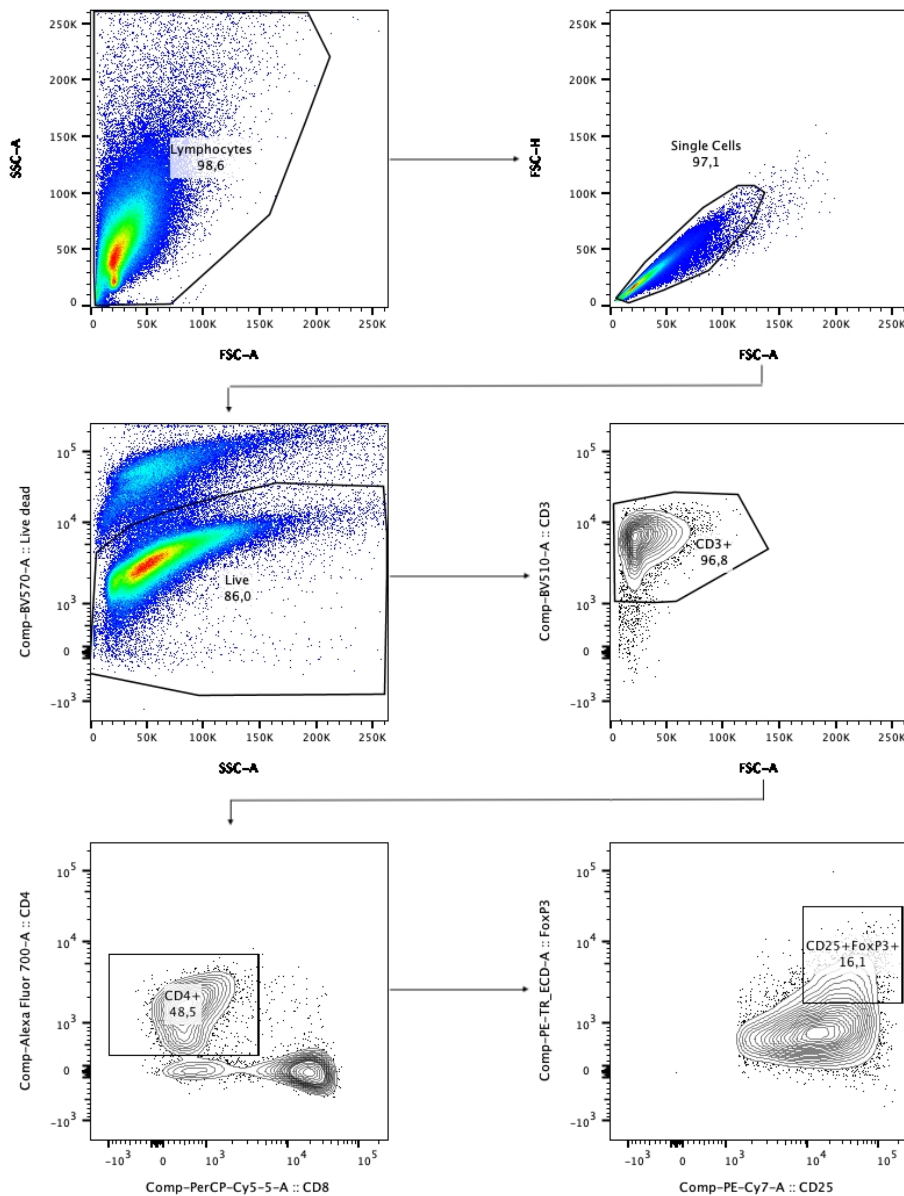

Figure 13: Gating strategy I. Lymphocytes, singlets, live, $CD3^+$, $CD4^+$, $CD25^+$ $FOXP3^+$ cells. *The first gating included all cells present, both live and dead, using an FSC versus SSC plot. Further the gate was set to singlets, using an SSC versus FSC plot. The next gating was for live cells, thus making sure that no dead cells were present in the analysis, by the use of an SSC versus live/dead marker plot. From the live population it was gated for $CD3^+$ cells, ensuring the presence of T-lymphocytes. Further gating involved separating $CD4^+$ cells from $CD8^+$ cells, before gating on $CD25^+FOXP3^+$ cells.*

Within the $CD4^+$ population it was gated for different T-cell markers (Fig. 14). These markers were CD25, CD45RA and CTLA4. Expression of CD45RA, CTLA4 and other markers within $CD4^+$ population did not give significant results within and between patients and controls, and the results are thus not shown.

From singlets →live → CD3+ →CD4+ gating

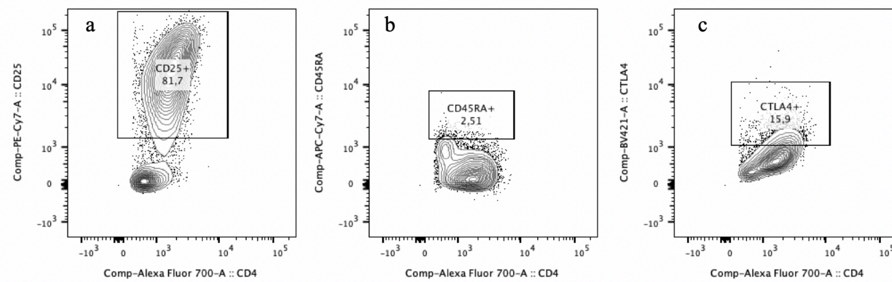

Figure 14: Gating strategy II. Lymphocytes, singlets, live, $CD3^+$, $CD4^+$ cells expressing (a) $CD25^+$, (b) $CD45RA^+$ and (c) $CTLA4^+$. *From the CD4+ population it was gated for different T-cell markers of interest. These include, from the top left: CD25, CD45RA and CTLA4.*

Within the population of $CD25^+$ cells expressing FOXP3, it was gated for markers of T-cells and Tregs (CD31, CD39, CD304, CTLA4 and Helios) (Fig. 15).

From singlets →live → CD3+ →CD4+ → CD25+ → FOXP3+ gating

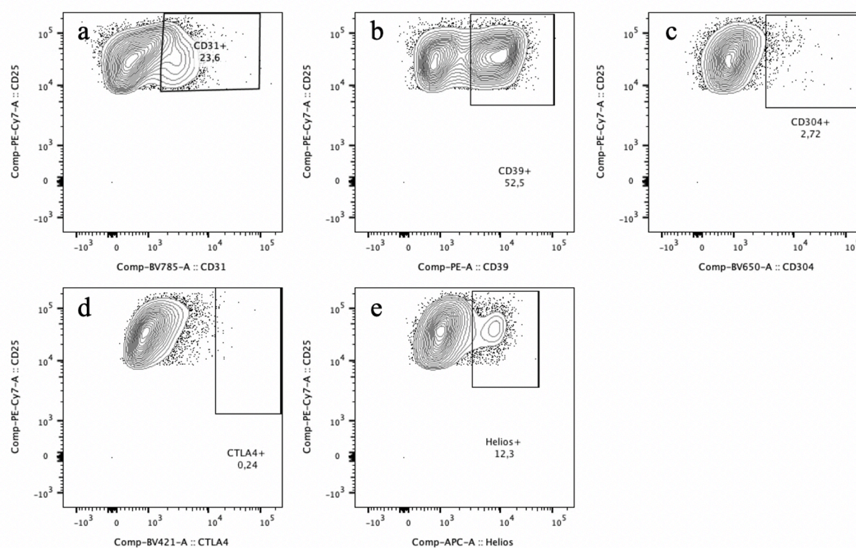

Figure 15: Gating strategy III. Singlets, live, $CD3^+$, $CD4^+$, $CD25^+$, $FOXP3^+$cells expressing (a) CD31, (b) CD39, (c) CD304, (d) CTLA4 and (e) Helios. *Within the FOXP3 positive cell population the gating is again set to intra- and extracellular Treg markers, making sure that one has managed to characterize the wanted cell population. These markers include CD31, CD39, CD304, CTLA4 and Helios.*

4.5.2. Live cells

We plated 200000 cells (PBMC) per well and were left with different cell numbers after cell harvesting and staining. This was seen in the flow cytometry results, where a difference in the fraction of live cells between the samples was observed. The following figure shows one representative flow figure for one of the patient samples (patient 5) (Fig. 16 (a)). From the diagram in Fig. 16 (b) it is further seen that the overall number of live cells, for both patients (no drug: 57.700±25.492, drug: 43.580±25.015) and controls (no drug: 64.360±15.333, drug: 57.960±11.835) were highest for the samples not treated with abatacept. A statistically significant decrease in the fraction of live cells was found within the patient population after the addition of abatacept (p-value 0.0018), supporting a trend towards abatacept reducing the live cell fraction (Fig. 16 (c)). All control samples, with the exception of control 2 who showed a slight increase, had a lower frequency of live cells post-treatment with abatacept, but significance was not obtained (p-value 0.1401) (Fig. 16 (d)).

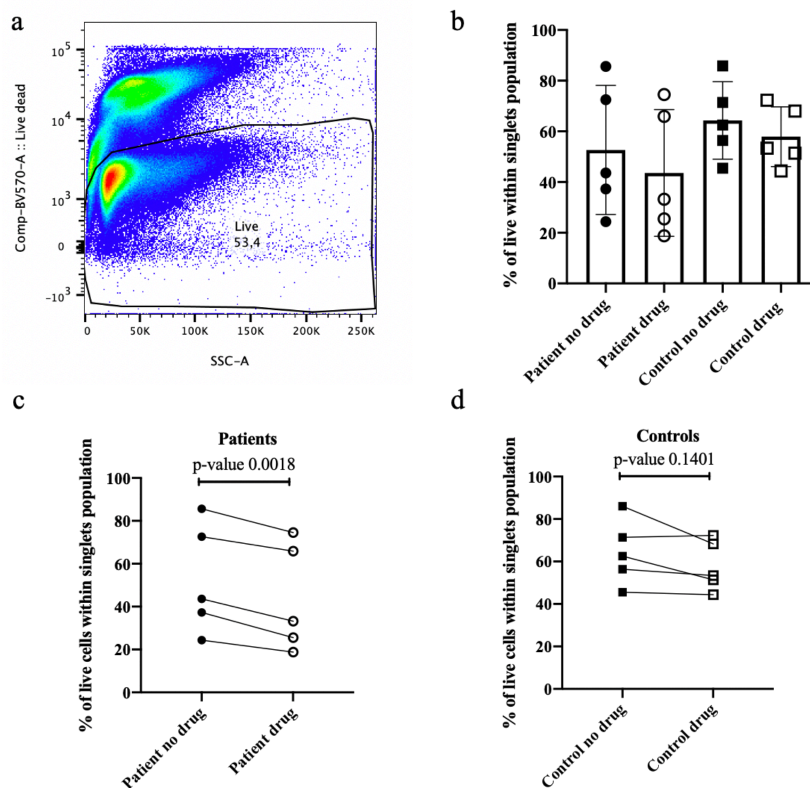

Figure 16: Live cells. *(a) Shows an example of what the live fraction might look like. Here, patient 5 is representative for all samples. (b) The fraction of live cells within the single positive cell population for both patients and controls. Live cell fractions vary from under 20 to over 80 when looking at frequency of parent. The boxes give the mean values, while the error bars represents standard deviation. Statistical analysis on differences between the two groups (patients and controls) can be found in table 8. (c) Comparison of patient no drug and patient drug samples. A significant p-value of 0.0018 was obtained when performing a paired parametric t-test between patients no drug and drug. (d) Comparison of control samples with and without abatacept. Statistical significance was not found within the control population (p-value 0.1401) by using a paired parametric t-test.*

4.5.3. Cell survival and proliferation

We investigated how the cells divide within the $CD4^+$ population, by using the proliferation index. Staining the cells with CFSE gave us the opportunity to track the rounds of cell division (141), where the proliferation index represents the mean number (142), and was here analyzed by FlowJo. We looked for significant differences between the no drug and drug samples for both patients and controls. It was observed that the cells had a lower frequency of division when treated with abatacept in both groups (Fig. 17). Patient cells were not dividing as frequently when drug was added, compared to the no drug samples (Fig. 17 (a)). The exception was patient 5, where a small increase in proliferation was observed after the addition of drug. Differences in proliferation between patients no drug and drug were found to be significant (p-value 0.0462). Unlike the patient samples, all control samples showed a decreased proliferation index when treated with abatacept, but no significance was obtained (p-value 0.1007) (Fig. 17 (b)).

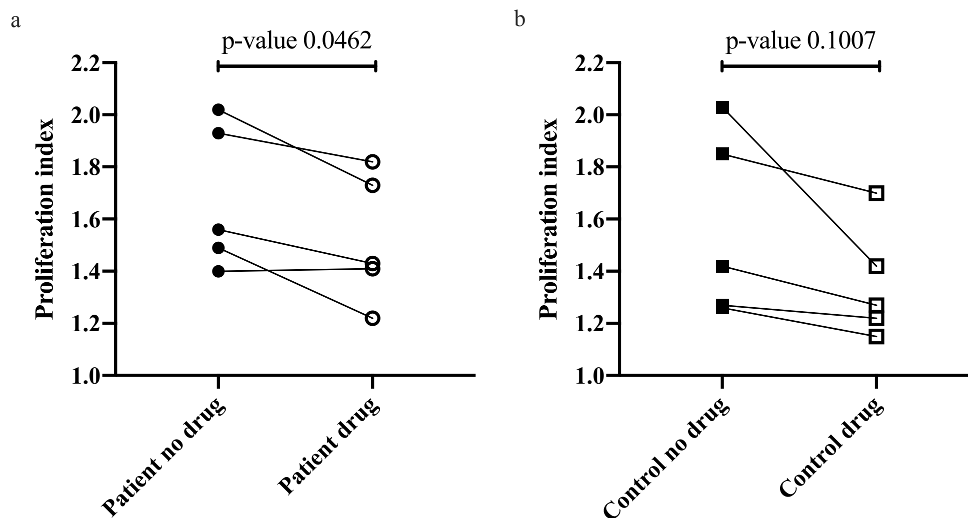

Figure 17: Comparison of proliferation indexes within (a) patient and (b) control populations. *A measure of cell division was given by the proliferation index. The results indicated, with significance (p-value 0.0462), that the patients' cells had a lower frequency of division when treated with abatacept. For the controls, all samples showed a decreased rate of division when abatacept had been added, but the difference before and after treatment was not statistically significant, with a p-value of 0.1007. P-values were obtained by performing a paired parametric t-test between patients no drug and drug, and between controls no drug and drug.*

When looking at cell proliferation, the results did not indicate any difference in the division rate between patients and controls no drug and drug, even though the patients (no drug: 1.680 ± 0.277, drug: 1.522 ± 0.247), in average, seemed to have cells dividing at a higher rate than the controls (no drug: 1.566 ± 0.353, drug: 1.352 ± 0.218) after the addition of abatacept

(Fig. 18 (a) and (b)). No significant differences were found when comparing patients and controls no drug (p-value 0.5476), and patients and controls drug (p-value 0.2460) (Fig. 18 (a) og (b)).

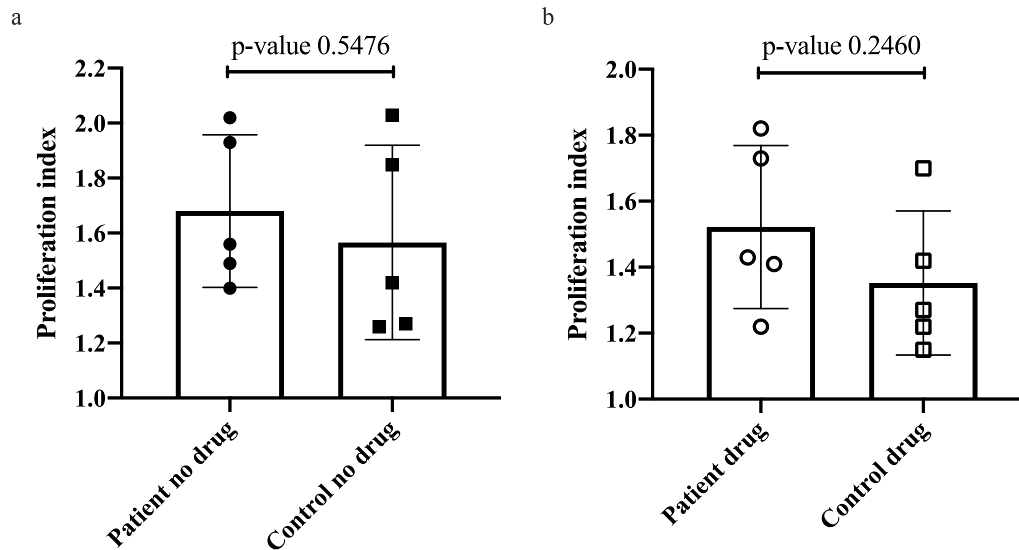

Figure 18: Comparison of proliferation indexes from patients and controls. (a) Patients and controls no drug, and (b) Patients and control drug. *In average it seemed like the patients had cells with a slightly higher ability to divide after treatment, but there was not found significance when looking at differences between the patient and the control groups, both with and without the presence of abatacept. To examine differences between patients and controls no drug, and patients and controls drug populations, a Mann-Whitney unpaired, non-parametric t-test was used. The boxes represents the mean proliferation index and the standard deviation is given by the error bars.*

4.5.4. Activated cells

CD25 was used as a marker for T-cell activation (66) and the gating strategy for CD25 is shown in Fig. 19 (a). A flow figure is shown for patient and control 4 no drug and drug (Fig. 19 (a)). The fraction of activated cells decreased in the samples with abatacept added compared to the no drug samples for both patients and controls (Fig. 19 (b)). Statistical analysis gave significant results between patient no drug and patient drug (p-value 0.0425), but not for the controls (p-value 0.1411). The graph and numbers still gave a clear indication of a lower frequency of activated cells after treatment with abatacept, which was the case for all patients and controls, and for the patient samples supported by a significant p-value of 0.0425 (Fig. 19 (c)).

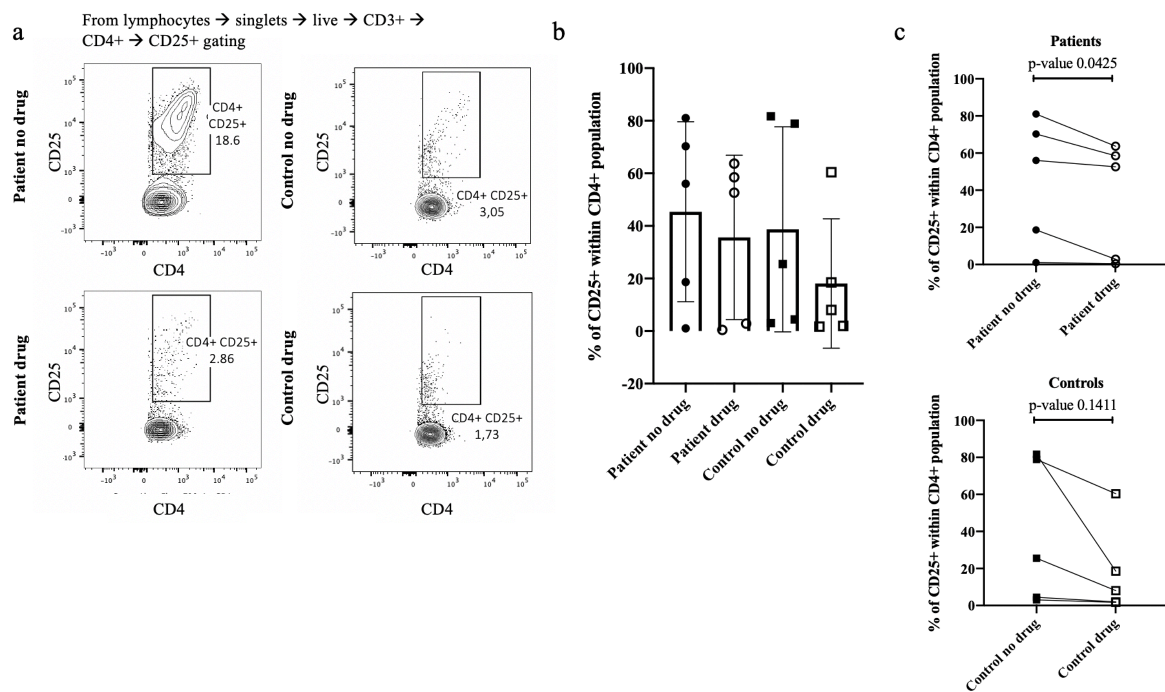

Figure 19: Frequency of CD4⁺ cells expressing CD25 (activated cells). *(a) Example showing the population of activated cells, here represented by patient and control 4 no drug (top) and patient and control 4 drug (bottom) (b) The fraction of activated cells within the CD4+ population for both patients and controls are shown, statistical data can be found in table 8. The boxes show the mean values of expression, while the standard deviation is represented by the error bars. (c) Comparison of patient and control no drug with patient and control drug, respectively. Statistical analysis (paired parametric t-test within patient and control populations) gave significant results for the patient population (p-value 0.0425), which was indicative of the frequency of activated cells being lower when treated with the drug. Significance was not found for the controls (p-value 0.1411).*

From the cells expressing CD25 (activated cells), we examined the expression of CTLA4 (Fig. 20). The representative flow figure included (Fig. 20 (a)) is from patient and control 5. The results indicated that the patients (no drug: 14.540±22.524, drug: 18.368±19.318, p-value 0.5487), in average, had a higher fraction of CTLA4 expressing cells after being treated with abatacept, compared to the no drug samples and to the controls (no drug: 5.280±3.387, drug: 10.918±13.452, p-value 0.3605) (Fig. 20 (b)). Statistics performed between patient no drug/drug and between controls no drug/drug, did not lead to significance (Fig. 20 (c)).

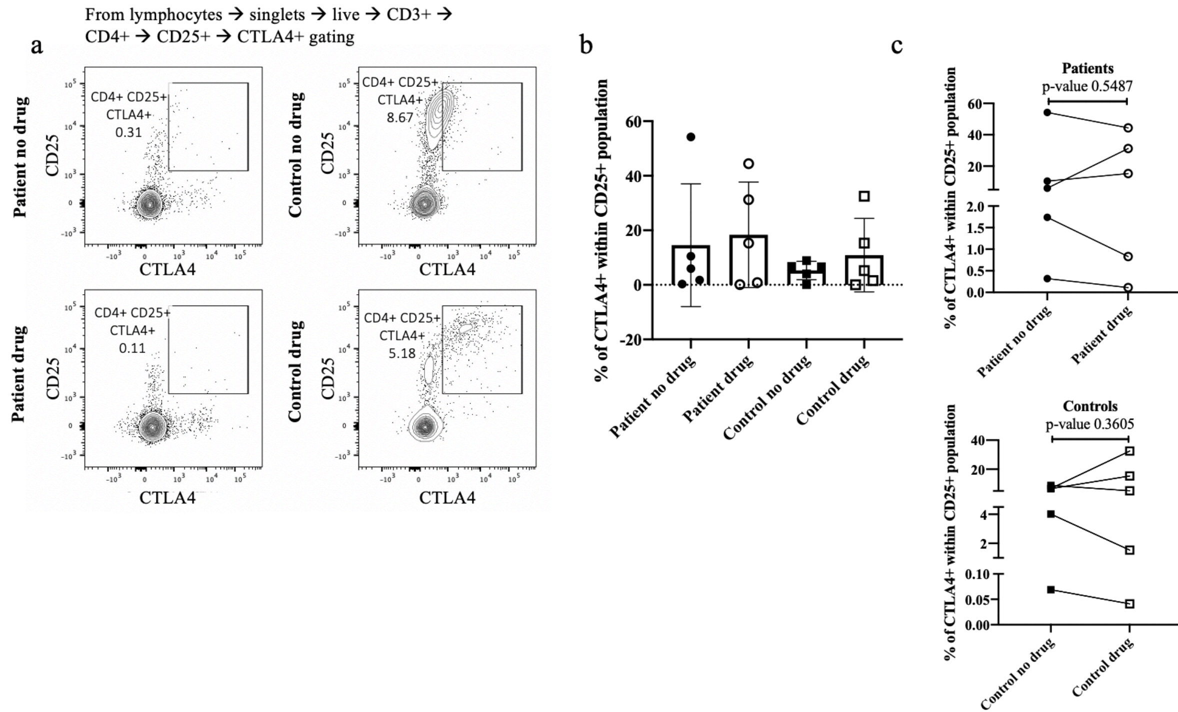

Figure 20: Frequency of CD25+ cell expressing CTLA4. *(a) Flow results showing fraction of CD25⁺cells expressing CTLA4, here exemplified by patient and control 5. (b) Overview of all sample parallels from both patients and controls. Mean values are represented by the boxes and the standard deviation by the error bars. For statistical analysis see table 8. (c) Comparison of CTLA4 frequency within patient and control populations respectively. Statistical analysis (Wilcoxon paired, non-parametric t-test within patient and control populations) did not lead to significant p-values, which were high for both patients and controls when comparing no drug and drug samples.*

We also examined the expression of CD31, CD39, CD304 and Helios in activated cells. A close to significant p-value of 0.0625 was obtained for CD31 in the patient population, and for CD39 a relatively low p-value of 0.0810 was obtained, also for the patient samples (Fig. 21 (a) and (b)). No significant results were found for the other markers and a variation in expression, both for no drug and drug, was observed (Fig. 21).

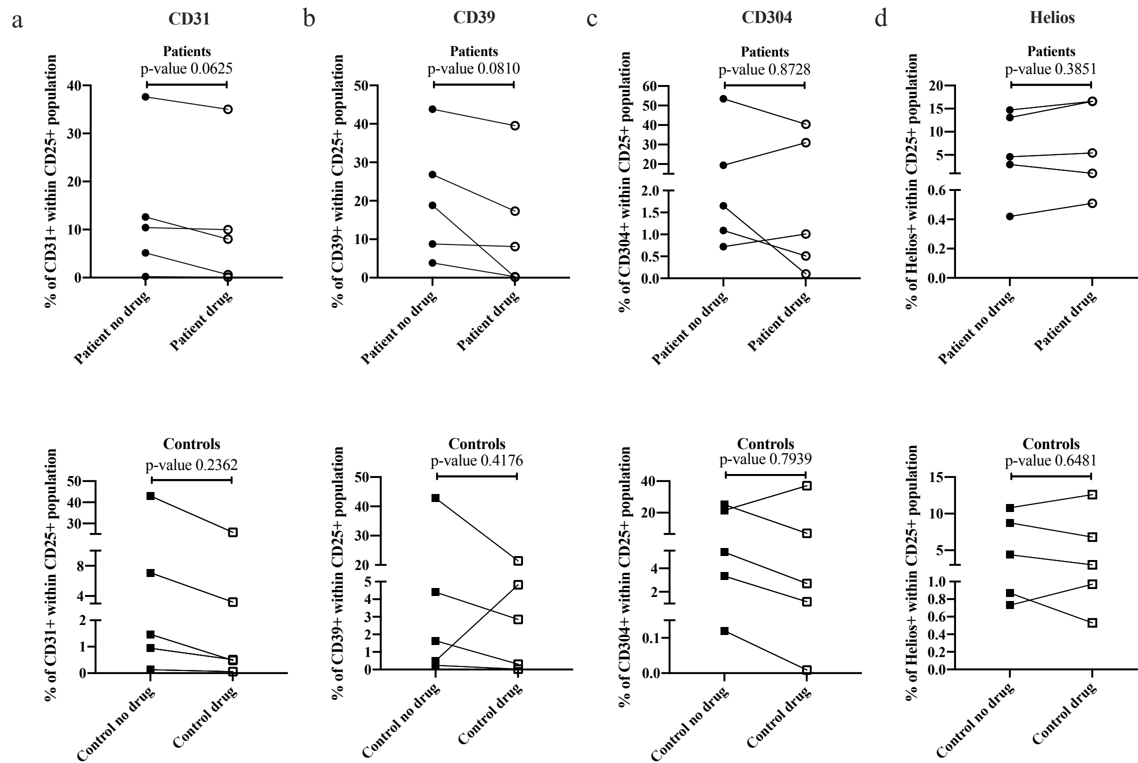

Figure 21: Expression of different T-cell markers in activated cells. *(a) CD31⁺ cells within CD25⁺ population patients (upper panel) and controls (lower panel). (b) CD39⁺ cells within CD25⁺ population patients (upper panel) and controls (lower panel). (c) CD304⁺ cells within CD25⁺ population patients (upper panel) and controls (lower panel). (d) Helios⁺ cells within CD25⁺ population patients (upper panel) and controls (lower panel). No significant differences were found between no drug and drug for neither patients nor the controls, when performing a paired parametric t-test within the two populations. Near significant differences were found for CD31 (patients) and for CD39 (patients), supported by p-values of 0.0625 and 0.0810 respectively.*

4.5.5. Tregs and CTLA4

From the population of $CD4^+$ cells expressing CD25, it was further gated on cells expressing FOXP3, i.e. Tregs (38). The results indicated a decrease in the FOXP3 expressing population for patient 1, 3, 5 and 6, while an increase was seen for patient 2 with the addition of drug, when compared to the no drug samples (Fig. 22). This was also the case when looking at the control samples; there was a decrease in the $FOXP3^+$ cell fraction in the presence of drug for control 2, 3, 4 and 5, while control 1 showed an increase. A flow figure is included, here represented by patient and control 5 (Fig. 22 (a)). Overall, patient samples (no drug: 22.010±21.947, drug: 19.224±21.643) had a higher fraction of FOXP3 expressing cells than the control samples (no drug: 14.562±10.584, drug: 10.552±9.434) (Fig. 22 (b)). There were no differences between patient no drug and patient drug (p-value 0.4838), as were the case for the control group (p-value 0.3489) (Fig. 22 (c)).

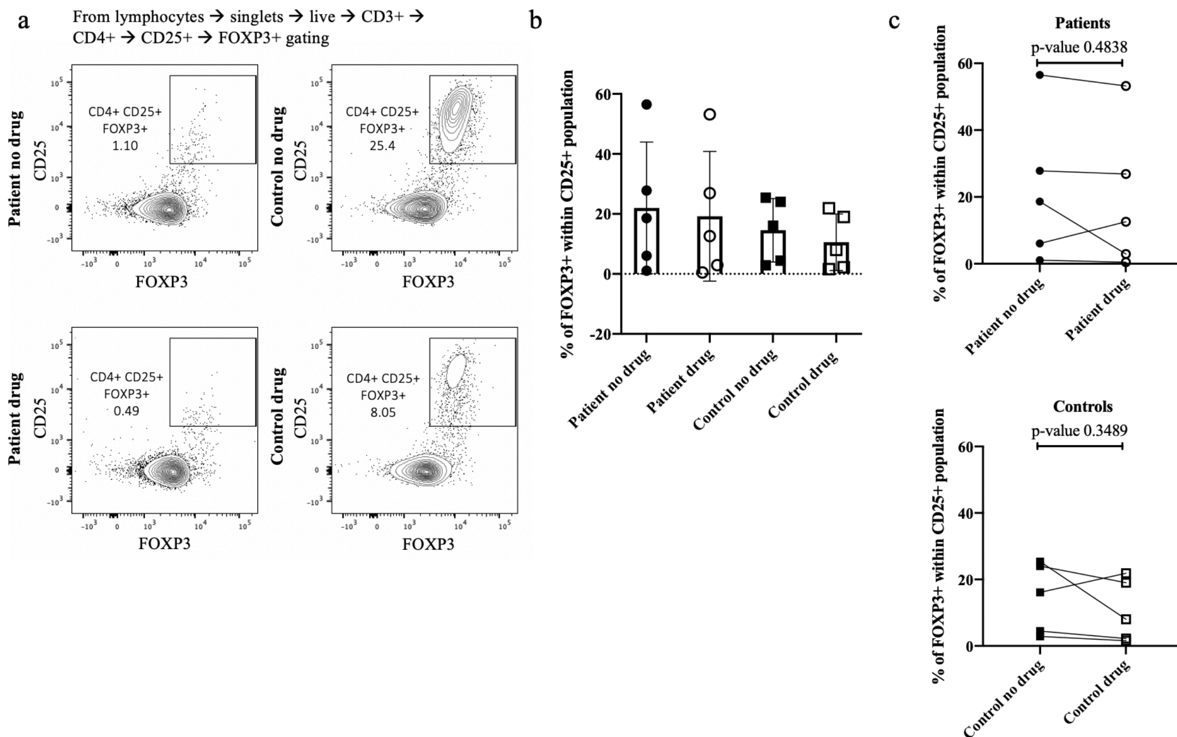

Figure 22: Fraction of CD4⁺ CD25⁺ cells expressing FOXP3 (Tregs). *(a) Fraction of CD25 positive cells expressing FOXP3, here exemplified by patient 5 no drug (top) and patient 5 drug (bottom). (b) The majority of the samples showed a decrease in the frequency of FOXP3 expressing cells when comparing no drug and drug samples for both patients and controls. Mean values are presented by the boxes, while the error bars show the standard deviation, for statistical analysis please see table 8. (c) For one patient sample (patient 4) and one control sample (control 3) there was an increase in the FOXP3 positive population when the sample was treated with abatacept. The results were considered not significant as no p-values less than 0.05 were obtained. Statistical analysis involved a paired parametric t-test performed between patients no drug and drug, and between controls no drug and drug.*

Within the FOXP3⁺ population it was gated for cells expressing CTLA4. Fractions of CTLA4 expressing cells within the Treg population are shown in Fig. 23, where a flow figure is included from patient and control 4 (Fig. 23 (a)). In general, the results indicated that the fraction of Tregs expressing CTLA4 was increased when going from no drug to drug for both patients and controls (Fig. 23 (b)). The exception was control 4 where a decrease was observed when comparing the no drug and drug samples. Statistical analysis gave a significant p-value of 0.0092 for the patient population, while no significance was found for the control group (p-value 0.1170), when looking at differences between no drug and drug. All samples from patients showed an increased expression of the CTLA4 on Tregs when treated with abatacept, a trend that was supported by a relatively low p-value of 0.0092 (Fig. 23 (c)).

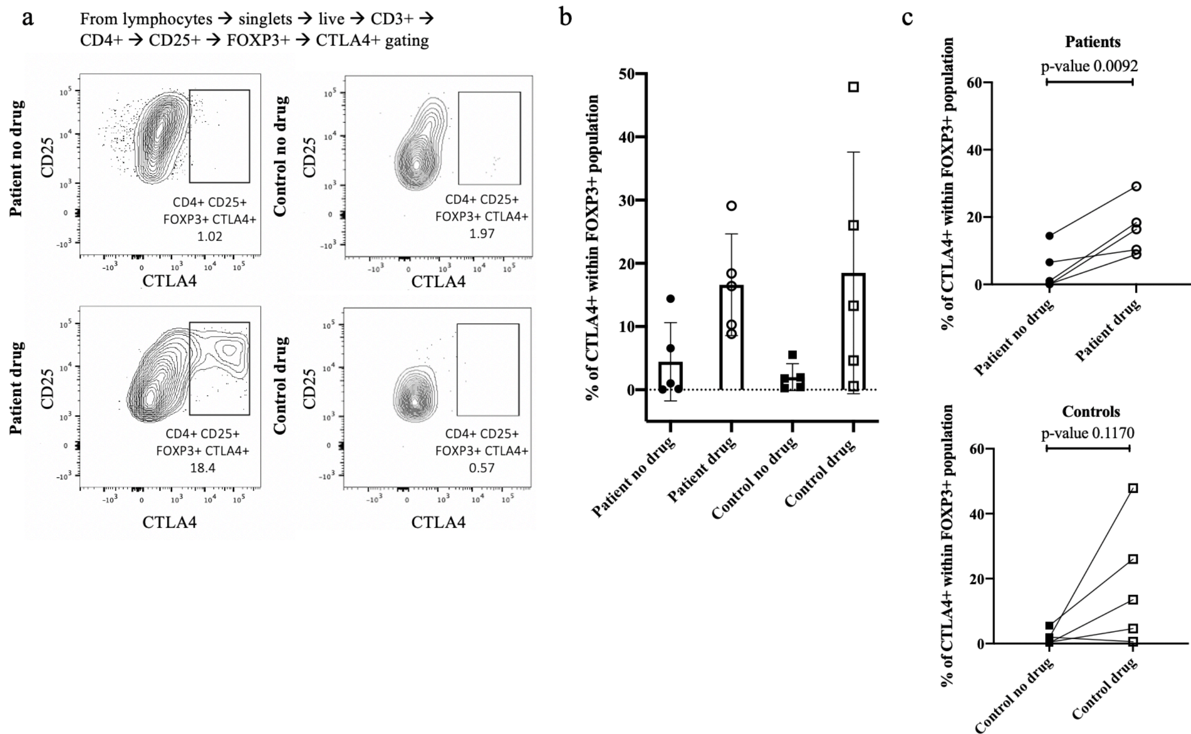

Figure 23: Fraction of FOXP3⁺ cells expressing CTLA4. *(a) Fraction of FOXP3 positive cells expressing CTLA4, here exemplified by patient and control 4 no drug (top) and patient and control 4 drug (bottom). (b) The majority of samples showed an in increase in the CTLA4 expressing population after the addition of abatacept, here represented by mean values (boxes) and standard deviation (error bars), in addition to the individual values. Statistical analysis can be found in table 8. (c) Comparison of patient and control no drug with the samples added drug for all parallels. All samples, except for control 4, showed an increase in the fraction of FOXP3 positive cells expressing CTLA4 after abatacept was added. The results were considered not significant, as the p-value obtained from paired parametric t-tests between control no drug and drug, was over 0.05. For the patients, the differences were considered statistically significant, based on a p-value of 0.0092.*

Data for the remaining Treg markers (CD31, CD39, CD304, Helios) are shown in Fig. 24. The results showed great variation in the fraction of cells expressing the different markers, as it both decreases and increases, when comparing cells treated or not treated with abatacept. The exception was CD39 (Fig. 24 (b)), where the trend clearly indicated a decrease in protein expression from the no drug to the drug population for both patients (p-value 0.0387) and controls (p-value 0.0466), a trend supported by significant p-values.

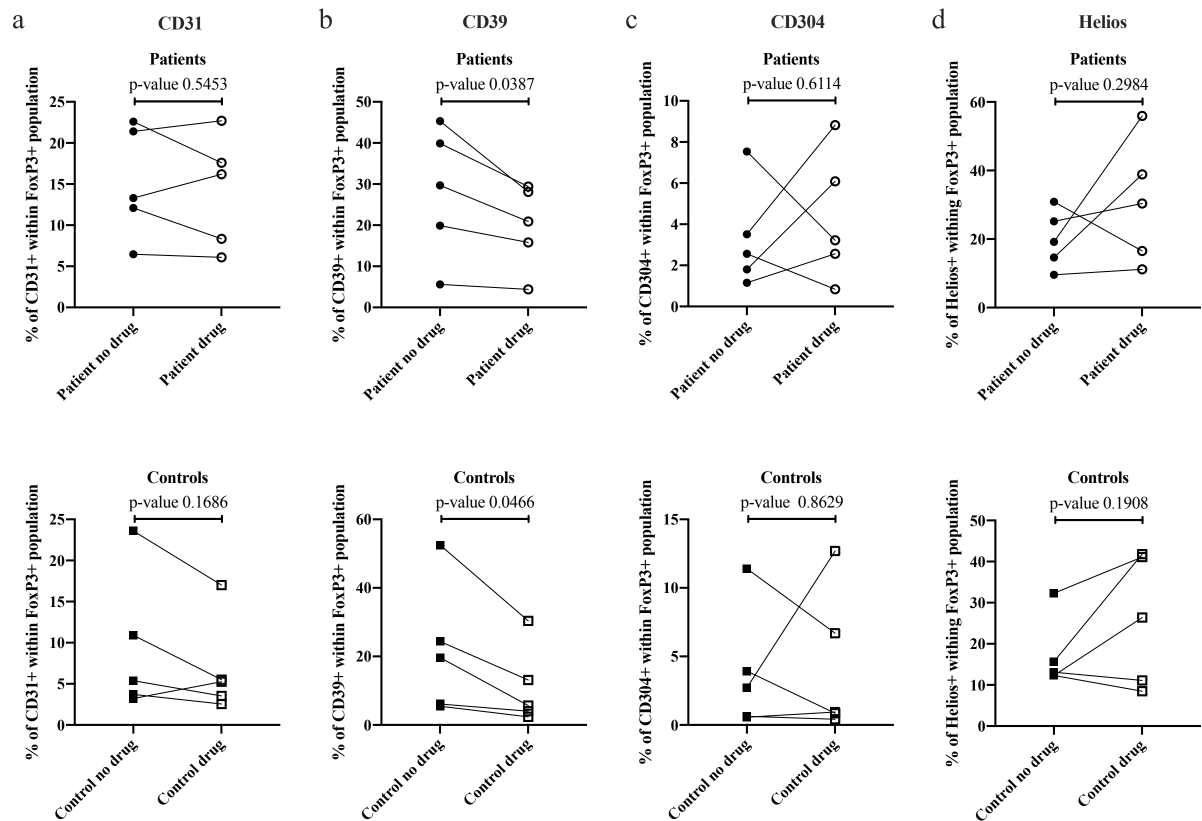

Figure 24: Cell expression of different Treg markers within the CD4$^+$CD25$^+$FOXP3$^+$ population. *(a) CD31 patients and controls, (b) CD39 patients and controls, (c) CD304 patients and controls, and (d) Helios patients and controls. The cells, treated or not with abatacept, showed a great variation in the expression of different Treg markers. No significant results were obtained, as most p-values were high. Most samples showed both an increase and a decrease in protein expression, when looking at both patient and control samples for most markers. CD39 was the exception. For this marker, the graph clearly showed a decrease in expression, for both patients and controls, when going from the no drug to the drug population. The observed trend is strengthened by significant p-values of 0.0387 and 0.0466 for patients and controls respectively. Paired parametric t-tests were performed between patients no drug and drug, and between controls no drug and drug samples, to look for significance.*

To further examine differences between patients and controls for some of the cell markers, we normalized the data by taking the difference between no drug and drug for each subject and within each group of patients and controls, respectively. After normalization, statistical analyses were performed between patient and controls for proliferation and different subpopulations of cells (live cells, activated cells, Tregs and CTLA4 within CD25$^+$ and FOXP3$^+$ cells), including Treg markers and CTLA4. No significant p-values were obtained (table 8) and thus there was no difference between the two groups.

Table 8: Statistics on normalized data between patients and controls.

Cell population	Average difference between no drug and drug (% point)		P-value
	Patient	Control	
Proliferation	0.16	0.21	0.8889
Live cells within lymphocytes, singlets population	9.1	6.70	0.5476
$CD25^+$ within $CD4^+$ population	9.76	20.58	0.4206
$CTLA4^+$ within $CD4^+$ $CD25^+$ population	-3.83	-5.64	0.8413
$FOXP3^+$ within $CD4^+$ $CD25^+$ population	2.74	3.88	0.5476
$CTLA4^+$ within $CD4^+$ $CD25^+$ $FOXP3^+$ population	-12.19	-16.52	>0.9999
$CD31^+$ within $CD4^+$ $CD25^+$ $FOXP3^+$ population	0.98	2.36	0.5476
$CD39^+$ within $CD4^+$ $CD25^+$ $FOXP3^+$ population	8.36	10.52	0.6905
$CD304^+$ within $CD4^+$ $CD25^+$ $FOXP3^+$ population	-0.99	-0.47	0.6905
$Helios^+$ within $CD4^+$ $CD25^+$ $FOXP3^+$ population	-10.69	-8.64	>0.9999

4.6. IFN-γ production in cells treated or untreated with abatacept

A sandwich ELISA was performed on the supernatants from the activated cells, treated or not with abatacept, in order to detect expression of IFN-γ. The concentration of IFN-γ was determined by measurement of the absorbance at 450 nm and by the generation of a standard curve (Appendix V). IFN-γ concentration in each sample, adjusted for dilution factors, was provided directly by the software used for analysis. Samples not falling within the range of the

standard curve was further diluted and run in a second ELISA assay.

The results indicated that the patients had a higher production of IFN-γ (no drug: 18160±31387 pg/mL, drug: 20155±36651 pg/mL), compared to the control samples (no drug: 7763±12945 pg/mL, drug: 2030±3432 pg/mL), both with and without the addition of abatacept (Fig. 25). For all patient and control samples, except patient 2, the concentration of IFN-γ was decreased when the cells were treated with abatacept (Fig. 25). It was further observed that patient 2 had a very high IFN-γ concentration, compared to the other samples (Fig. 25 (a)). This sample was not within the range of the standard curve, but it is still presented and considered in statistical analysis. Given that the kit used has its limitations in accepted concentration values, the concentration of this sample could be even higher than reported here. Patient 5 drug and control 3 drug also fell outside the range of the standard curve, with no detectable concentration of IFN-γ and their concentration values were set to zero, and included in the statistical analysis. No significance was found when examining differences between no drug and drug within the patient (p-value 0.4049) and control (p-value 0.2364) populations respectively.

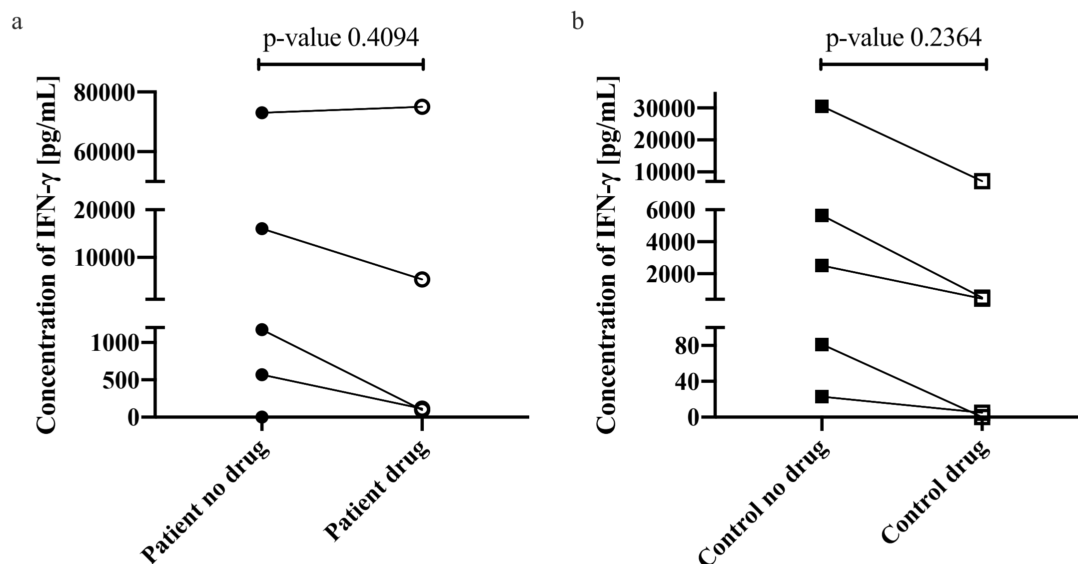

Figure 25: Concentration of IFN-γ in (a) patient and (b) control samples. *For the majority of samples, from both patients and controls, the concentration of IFN-γ decreased when the cells were treated with abatacept. All five control samples had a lower concentration of IFN-γ when treated with abatacept, but for control 3 no drug the concentration was too low to fit within the range of the standard curve. Patient 2 showed a much higher concentration than all other samples and also gave a small increase in concentration with the addition of abatacept. The production of IFN-γ seemed to be higher in the patient samples, compared to controls. Statistical analysis examining differences within the patient and control samples (paired parametric t-tests) did not lead to significance for neither patients (p-value 0.4049) nor controls (p-value 0.2364).*

Due to the large variation in the concentration of IFN-γ, within and between patient and control populations, we further normalised the values by using the differences between the no drug and drug samples within the patient and control population, but no difference was found between groups (p-value 0.4206). Patients had a mean difference in concentration of 4056.83 pg/mL, while controls had a mean difference of 7363.59 pg/mL, indicating that the overall amount of IFN-γ was larger in the no drug population for both patients and controls. We also compared patients and controls no drug, and patients and controls drug, without finding significant differences (Fig. 26). All samples were used in the statistical analysis and presented in Fig. 26, despite being outside the range of the standard curve.

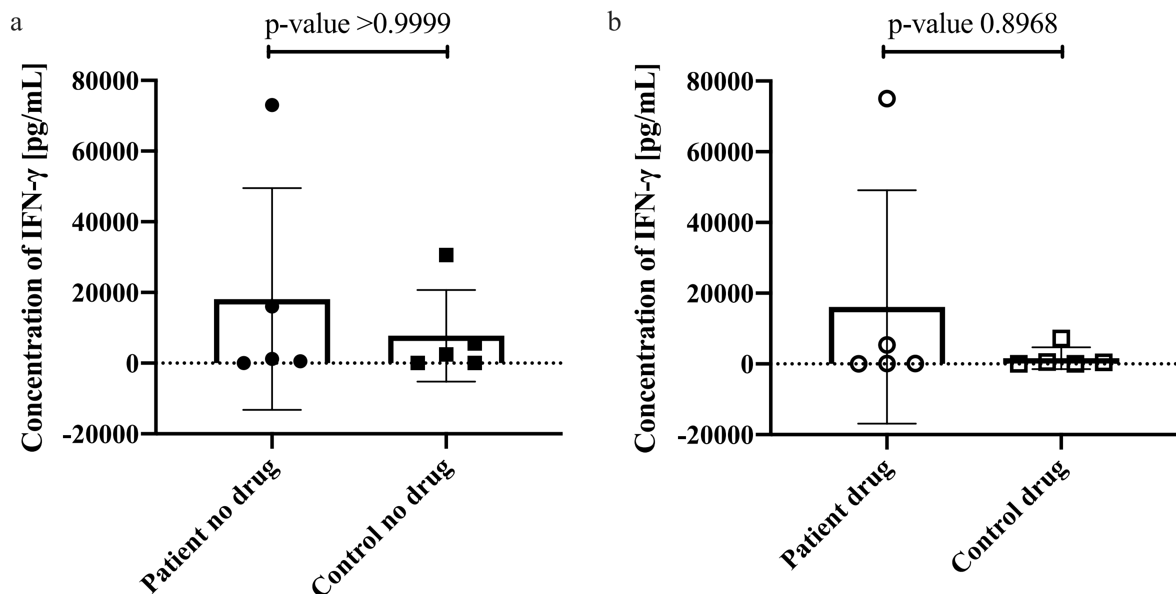

Figure 26: Differences between patients and controls no drug, and patients and controls drug. *To search for differences between no drug and drug we compared patient and control samples within the two populations. No significant differences were discovered, but the patients seemed to have a higher IFN-γ concentration both before and after treatment with abatacept, compared to the controls. For both patients and controls, the levels of IFN-γ were highest without the addition of abatacept. Statistical analysis looking at differences between the two populations (Mann Whitney unpaired, non-parametric t-tests), did not lead to a significant result. Here, the boxes represent the mean IFN-γ concentration values, while the error bars show the standard deviation.*

5. Discussion

5.1. The effect of abatacept on T-cells in patients with AAD can be screened by the method developed

We aimed to develop a method for *in vitro* drug screening in AAD patients at the KG Jebsen Center for Autoimmune Diseases to search for new treatment options, as these patients suffer from, amongst other, lower quality of life due to the unphysiological replacement therapy (126-128). The assay developed included treating cells from AAD patients and healthy controls with abatacept, growing them in cell culture for five days, staining with T-cell and Treg markers, and examine cell protein expression by flow cytometry, the production of IFN-γ by ELISA and transcription of selected genes by qPCR. We observed that the cells were able to divide and expand *in vitro* and results from flow cytometry showed decreased proliferation for the majority of samples post-treatment with abatacept. All together, these observations indicated that the developed method is suitable for *in vitro* drug analysis. Even though the assay proved successful, it was quite time consuming, raising questions about the efficiency. From start to finish it took almost a week to obtain results. Here, we also screened the effect of only one possible drug (abatacept) in one autoimmune disease (AAD) and method optimization is necessary before large-scale screening could be a possibility.

Our study had some limitations. The effect of abatacept was examined in only five AAD patients and five healthy controls, while six APS-1 patients, one AAD patient and seven healthy controls were used in qPCR involving non-activated cells, in the search for candidate genes. APS-1 patients involved in the study were all suffering from AAD, which is one out of three criteria for diagnosis (34), and was also the rationale for including them. The low number of patients and controls gave the study a low statistical power and the inclusion of more test subjects would have been beneficial. Thus, we have to use caution when interpreting the results of the statistical tests used. Patients were chosen to participate without taking the duration of AAD into consideration. We do not know if this affected how they respond to abatacept treatment, as it has not yet been examined in these patients, but in type 1 diabetes, the effect have been studied in patients with recent-onset disease (122, 123). All controls were age- and sex-matched with the patients, and we sat an age limit of ± 10 years for the controls. Even though autoimmune diseases are known to have a higher prevalence in female than in male, the prevalence is close to the same for both sexes in AAD (female:male ratio 1.5-3.5:1) (103, 143, 144). Despite this, we still chose to sex-match the controls, thus taking biological differences

that could be caused by age and sex into consideration.

We have been working with human biological material and did not have continuous access to sample material. PBMC from patients were obtained from the ROAS registry, while PBMC from healthy controls were isolated from fresh blood samples obtained from the blood bank at Haukeland University Hospital. Both patient and control samples were stored at -150°C until use. It was observed that the number of cells obtained from each sample fluctuated greatly, most likely due to biological differences between individuals, as some individuals might have a higher/lower white blood cell count than others (145), or it could be due to faults in experimental techniques, such as pipetting. To try to improve the number of cells, one possibility would have been to use Ficoll-Paque density centrifugation media instead of the CPT Vacutainer tubes for PBMC isolation, but a study by Corkum *et al.* have shown that the two methods are equally efficient (146). We had no available information about neither patients nor controls at the time of blood testing, except for sex, age and, for the patients, a diagnosis. We did not know if the subjects had an ongoing infection or if they were recovering from a recent infection, if they were on any medications, or if the blood was drawn fasting. Also, we did not have information about the treatment the patients were receiving or what time of day the samples were taken. All factors mentioned above could have affected the results (147, 148).

During the experiment, we also experienced problems with cell-loss, which might be caused by freezing and thawing of samples and/or several washing and transferring steps, all increasing the probability for loss of product (147). In addition, we observed variations between the assays run on different days. This was especially seen in the live cell fraction, which decreased by a considerable amount from assays 1-2 to assays 3-5, with indications of a larger decrease amongst the patients (Fig. 16 (c)). We also saw, in flow cytometry, that some of the populations "moved" in the last assays, when compared to the first ones. We do not know why this occurred, as all cells were treated equally in sample preparations, cell culture and analysis by flow cytometry. The only difference between the assays was how the plate coated with anti-CD3 was handled. For assays 1-2 the plate was coated and incubated at 37°C and 5% CO_2 for two hours, while for assays 3-5 the plate was coated and sealed one day prior to the experiment, and stored at 4°C overnight. We do not know for certain if this has affected our results, but as the plate was properly sealed, we believe that a possible effect was minimal. We chose to let the cells grow and expand in culture for five days, in order to examine cell proliferation. The cells were treated with abatacept once at day one and after this treatment was discontinued. At day three the cells

were split and given more medium, thus ensuring that they were given enough room to continue expanding. We questioned if abatacept should have been dosed more than once during the five days, but given that abatacept has a half-life ranging from 8-25 days, with an average half-life of 13.1 days (117), this was considered not necessary. Also, we were concerned that a higher dose and/or a higher dosage interval could lead to an increased frequency of cell death, as we had a limited number of cells to begin with.

5.2. The expression of genes involved in the T-cell signalling pathway was overall low in resting cells from both AAD patients and healthy controls

A commercial SYBR green qPCR assay was used to examine differences in gene expression between six APS-1 patients, one AAD patient and seven age- and sex-matched healthy controls, in genes related to CTLA4-function. No significant differences were found between the groups. RNA samples used were obtained from the ROAS registry, all with RIN-values indicating high quality RNA (140). The qPCR assay used in this project gives 96 different genes for use in analysis (84 after removal of housekeeping genes and control-probes), and one of the most interesting in this project is *CTLA4*. This gene has been shown to be involved in susceptibility for developing AAD (115, 116) and is also known to be an important component in the function of Tregs (46, 89). In addition, a recent GWAS has shown that polymorphisms in the *CTLA4* gene predispose for development of AAD (Eriksson, Røyrvik and Aranda-Guillen *et al.*, submitted manuscript). From the results (Fig. 10), one can see that *CTLA4* has a low expression in both patients and controls. This is not a surprise, as it is known that *CTLA4* is expressed after the T-cell has received a signal from the APC and a following co-stimulatory signal from the CD28-CD80/86 interaction (97). To obtain a clearer view of the expression of *CTLA4* and other genes on the plate, the experiment could have been done on activated cells. However, the purpose of this experiment was to select genes of interest to further study in activated cells, as we knew the limitation in sample size would only allow us to investigate the gene expression of a selected handful of genes.

The initial qPCR assay did not reveal many genes that reached significance when comparing expression levels between patients and controls, but the fold change still indicates a difference in the expression of certain genes in patients and controls. A p-value of 0.039654 was found for *FASLG*, a gene encoding a protein involved in both thymic and peripheral apoptosis (30-32), and that was downregulated in the patients, when compared to the controls (Fig. 10). Due to multiple testing, the limit for significance was lowered to 0.01, and the change seen for

FASLG was thus not considered significant. From Fig. 10 it is evident that there is a fold change value indicating differences in expression of some of the interleukins; *IL13* and *IL10*. We speculate that this might be caused by an ongoing infection or that one of the subjects were recovering from an infection, as these cytokines, in different ways, are involved in an immune response (149, 150), but this information was not available. Also, there was a variation in the sample size populations; for *IL10* only five patients and two controls are examined for differences in gene expression, and the involvement of more patients and controls are necessary to validate the findings. Since we used a SYBR green qPCR assay, a melting curve analysis could have been performed to ensure that only the specific wanted product was present in each sample (151). However, we chose to rely on the quality of the commercial assay used, hence no melting curve analysis was done. In general, the differences seen in gene expression might not be affected by AAD, but could be due to biological differences between individuals and it would have been interesting to examine a larger population. Since the difference was found in resting cells, and there is a continuous supply of patient blood samples to ROAS, this would be relatively easy to do. To see if there actually is a significant difference or biological relevance in gene expression, one has to select the genes with the most promising fold change and run further analysis to confirm the results.

5.3. The expression of candidate genes in activated cells, treated or untreated with abatacept, was in general low in both AAD patients and healthy controls

When the gene-expression assay was repeated on activated cells, treated or untreated with abatacept, from five AAD patients and five healthy controls, a TaqMan qPCR assay was used to explore effects on gene transcription on the selected genes from the resting cell study. We did not find significant differences in gene expression of the selected genes *CTLA4*, *FASLG, ICOS* and *JUN* within or between the patient and control populations (Fig. 11 and 12). The majority of signals appeared late in the qPCR cycle, indicating low gene expression. Fold change values did not give significant differences in gene expression after treatment with abatacept (Fig. 12), but patient 5 stood out from the remaining samples by showing a higher expression of *FASLG, ICOS* and *JUN*. Due to the involvement of *FASLG* and *ICOS* in the T-cell mediated immune response (31, 152), a higher expression in patient 5 might be caused by an infection at the time of sampling.

Concentrations of RNA in each sample was relatively low and the quality, assessed by the RIN-value, varied (Fig. 9 and appendix IV). This could be caused by the presence of genomic DNA

or the presence of RNases causing degradation of RNA (153), and also because the concentrations of the samples challenged the detection limit of the instrument. Due to sample size limitations, we had to use the RNA available, regardless of RIN-values or low concentrations. A premade TaqMan qPCR assay were chosen based on higher specificity compared to a SYBR green assay (154), and because they were pre-tested by the manufacturer. We chose to add the housekeeping gene (*B2M*) to each well, together with the TaqMan enzyme, probes (*CTLA4, FASLG, ICOS, JUN*) and the samples. The housekeeping gene amplified well in all samples, thus indicating a functional assay. Despite this, we still experienced problems with signals appearing late in the qPCR cycle for the genes of interest. We suspect that this could be due to low input RNA or a general low expression of the genes tested, but could also have been caused by problems with the probes. Cells used in this qPCR assay were taken from specific wells on the plate used for cell culture, and therefore, not only T-cells, but also dead cells and all other cells present in a particular sample, were collected. A possibility could have been using a type of flow cytometry known as fluorescent-activated cell sorting (FACS) to separate live cells, live $CD4^+$ cells or even $FOXP3^+$ Tregs (132), but this would require much higher cell numbers than obtained here.

5.4. Treatment with abatacept affected proliferation, the fractions of live cells and cell protein expression of T-cell/Treg markers

Flow cytometry was used to examine cell protein expression and the effect of abatacept on T-cells/Tregs in AAD. It was decided to use an abatacept-concentration of 20 μg/mL, as this concentration showed greatest effect of abatacept in pilot experiments. We saw that treatment affects both the fraction of live cells (Fig. 16) and proliferation (Fig. 17 and 18), with statistical significance for patients, but not for the controls. Also, we did not find differences between the two groups (patients and controls) for any markers of T-cells/Tregs (table 8). Our results indicated an overall lower frequency of division when cells were treated with abatacept, in line with findings in other studies (119, 155). The exception was patient 5, where a small increase in proliferation was observed post-treatment with abatacept. It is difficult to explain why this patient stood out, but we do know that it was most likely not due to degradation, as abatacept was stored in aliquots to avoid freezing and thawing. Patient 5 also differed from the other samples in the qPCR experiment on activated cells (Section 5.3), but we do not know if increased expression of *FASLG*, *ICOS* and *JUN* is connected with a slightly increased proliferation, after treatment with abatacept. As expected, a decrease in the amount of activated cells was observed, as the mechanism of action of abatacept is to inhibit/modulate T-cell

activation by interfering with the required co-stimulatory signal (Fig. 19) (117). Also, we used the same gating strategy for all samples, and aimed for equal gating for each marker within populations analysed at the same time/day, meaning that the same gate was set for CD4 for patients and controls no drug and drug. This might have caused a higher frequency of some subpopulations, for instance for $CD4^+CD25^+$ cells (Fig. 19 (a)), which make up approximately 10% of the $CD4^+$ cells, while roughly 1-2% these cells have been found to express high levels of CD25 and show suppressive properties (67, 156).

We defined Tregs as $CD4^+CD25^+FOXP3^+$ cells, as it has been shown that FOXP3 is important for their development and function (54). We hypothesized an increase in the amount of Tregs post-treatment with abatacept, in hopes of suppressing the autoimmune reaction, but we observed variation between samples, both for patients and controls, as treatment with abatacept resulted in both a higher and a lower frequency of Tregs (Fig. 22). Studies have shown that treatment with abatacept/CTLA4-Ig can cause both an increase and a decrease in the number/frequency/expression of regulatory T-cells (120, 121, 155, 157), and the results obtained here are therefore in line with previous reports, although inconclusive. A decrease in the fraction of Tregs could possibly be due to their dependency on CD28 for development and maintenance of homeostasis outside the thymus (43-45), as abatacept will inhibit signalling through CD28 by binding to its ligands CD80/CD86 on the APC (117). When examining the expression of different markers within the Treg population, we did not find significant differences between the groups (table 8), but CD39 showed an interesting decrease in expression for both patients and controls after abatacept treatment, reaching significance for both groups (Fig. 24 (b)). This might indicate a lower frequency of activated Tregs, as CD39 has been found to be especially expressed on activated Tregs (65). CD39 has also been suggested to be involved in the suppressive function of Tregs (69, 70) and a lowered expression could indicate altered Treg function as a result of abatacept treatment. More data and experiments involving a higher number of patients/controls are necessary to validate these findings, before drawing any conclusions.

Regardless of the frequency of Tregs, the protein expression of CTLA4 within this population increased for the majority of patients and controls after abatacept treatment, being statistically significant for patients (Fig. 23). We speculate that the increased expression might be due to the addition of a CTLA4-agonist. Despite an increased expression of the protein, the qPCR done on the same cells, revealed low expression of *CTLA4* (Fig. 11). If we had an unlimited

access to biological material, the experiments should have been repeated and included more patients and more cells, to further search for biological significant differences. Also, at the starting point for this study, the samples included had already been collected in ROAS, so they were not chosen optimally for this particular study. As of today, we do not know if these patients have malfunctioning or decreased numbers of Tregs, but interestingly defective Tregs have been found in patients with APS-1 and in patients with APS-2, and many of these patients also have Addison's disease (34, 158, 159).

5.5. Production of IFN-γ was altered after treatment with abatacept

Low expression of the *IFN*-γ gene was found in the initial qPCR experiment on non-activated cells, but the expression seemed to be higher in patients compared to controls (Fig. 10). However, we did not include *IFN-γ* further on in the analysis of expression level tests in activated cells because the differences between patients and controls did not exceed our "inclusion criteria". It has previously been shown that treatment with abatacept can cause lower expression of the *IFN-γ* gene in patients with rheumatoid arthritis (160), and RNA expression profiling in activated and treated/non-treated cells would have been interesting if we had had more material. Instead the production of the cytokine IFN-γ was investigated by an ELISA assay on the supernatant from activated cells, treated or untreated with abatacept, from five AAD patients and five healthy controls. We found that treated cells, in general, showed decreased secretion of the cytokine and the amount of IFN-γ was, in average, largest in the no drug samples for both patients and controls (Fig. 25 and 26), in line with the proposed mechanism of action of abatacept (117) and findings in other studies (119). This also corresponds to the overall decreased rate of proliferation discovered after treatment with abatacept and that the T-cells seemed to be more "active" within the patient population compared to the control samples.

Levels of IFN-γ in the samples examined were much higher than expected, as some of them fell outside the range of the standard curve even with a 1:100 dilution. This might be due to the addition of anti-CD3 when preparing the cells for analysis, as this will initiate the signalling cascade and activate the cells (20, 161). The concentration could also be higher, for some samples, than reported here, as the assay used had a detection limit determined by the standard curve (appendix V). We did not know whether patients and controls had an ongoing infection or if they were recovering from a recent infection at the time of blood testing, but this could

have affected the results, as IFN-γ is involved in the immune response by mediating differentiation of effector T-cells and macrophage activation (61, 64).

5.6. Conclusions

In summary, we have developed a method that can be used to screen the effect of different drugs on T-cells in patients with AAD at the KG Jebsen Center for Autoimmune Diseases, with the drawback of being quite time consuming. We have seen that abatacept affects cell survival, with the frequency of live cells, in general, being lower after treatment, reaching statistical significance for the patient population. We also found that abatacept overall causes a decrease in proliferation by cell culture and flow cytometry. Treatment with abatacept showed both decreased and increased frequencies of Tregs when compared to non-treated samples, thus being inconclusive as it varied amongst patients and controls. The overall expression of genes involved in the T-cell signalling pathway in resting cells was low, in both patients and controls. When examining gene expression before and after treatment with abatacept in activated cells, the same was observed for the candidate genes *CTLA4*, *FASLG*, *ICOS* and *JUN*, in both patients and controls. Treatment with abatacept also affected the production of IFN-γ, which was decreased after treatment for the majority of patients and controls.

The study was limited by a low number of participants and the restricted access to biological material. We did not find statistical significance in either gene expression nor in the effect of abatacept on the majority of the different T-cell subsets and, in particular, Tregs. However, statistically significant differences were found in patients for CD39 and CTLA4 after treatment, both within the Treg population. For the controls, differences in the fraction of $CD39^+$ cells amongst Tregs reached statistical significance. We do not yet know whether these differences are biologically relevant and further studies are needed to be able to determine this.

In order to further search for biological relevant differences, validate findings and identify possible new treatment strategies, the involvement of more patients, method optimization, and screening of several other drugs are required.

6. Further work

As of today, no cure exists for patients with AAD, and we do not know if they have impaired function or decreased frequency of Tregs. We chose to examine the effect of abatacept using classical and well-established methods, such as flow cytometry, qPCR and ELISA, but could have complemented these methods with other protein-level assays, such as mass cytometry (CyTOF) (162). When higher cell numbers are available from patients and controls, a further approach could be to use fluorescence activated cell sorting (FACS) to separate different T-cell subpopulations (132), including Tregs, and maybe perform single cell sequencing on the 10x platform (163), to reveal subtle and stable changes due to abatacept/other drug influence. To increase statistical power, the assay developed should have been repeated with a higher number of study participants to be able to state whether abatacept biologically and relevantly affects Tregs and if patients respond differently to treatment compared to controls. It could also be interesting to divide patients into groups based on the presence of polymorphisms in the *CTLA4* gene and further examine the effect of abatacept. Another possibility would be to examine the expression of different interleukins, such as IL10, which is important for Treg function (37, 41), by performing for instance an ELISA assay, by flow cytometry or other multiparametric assays.

Since AAD has such a great impact on patients' lives, as they depend on life-long treatment and follow-up by the specialized health care system (112), it is of major importance that we continue to investigate and try to develop new treatment options. Finding a cure for autoimmune disorders, such as AAD, is difficult, as the disease most often has progressed too far upon the time of diagnosis (112). Early disease discovery requires screening of risk populations with autoantibody assays and genetic testing. This raises ethical questions regarding testing otherwise healthy individuals and how this affects their lives, as they might end up feeling sick/sicker while waiting for test results. Furthermore, as the screening tools can only predict a possibility that the patient will actually get the disease, it might also involve the question of whether we should start treatment or not in these persons. It is also important, now and in future research, to take ethical use of sample material into consideration, which we believe to have managed here, as one blood sample from each study participant has been used to generate a great amount of data (cells, RNA, cytokines). With this in mind, it is still important to try to find treatment options that can work to prevent disease complications and increase the quality of life for these patients.

7. References

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8. Appendix

8.1. Appendix I

Additional information about patients and controls 1-5.

Patient	Sex	Age	Diagnosis	Organ specific autoantibodies	AIRE mutation	IFN-w autoantibodies
1	F	34	AAD	21OH	No	Negative
2	M	53	AAD	21OH	No	Negative
3	M	35	AAD	21OH	No	Negative
4	F	25	AAD	21OH	No	Negative
5	M	61	AAD	21OH	No	Negative

*AAD, autoimmune Addison's disease; 21OH, 21-hydroxylase

Control	Sex	Age
1	F	37
2	M	53
3	M	28
4	F	28
5	M	54

Additional information about patients 6-12.

Patient	Sex	Age	Diagnosis	Classic triad	Other manifestations	Organ specific autoantibodies	AIRE mutation	IFN-w autoantibodies
6	F	†31	APS-1	A, C	G, V, N	21OH, 17OH, SCC, NALP5	c.769C>T/c.1242_1243insA	Positive
7	M	†58	APS-1	A, HP	Al	21OH, 17OH, SCC	c.22C>T/c.402delC	Positive
8	M	29	APS-1	A, C, HP	M, A, E, K	21OH, 17OH, TH	c.967-979del13/c.967-979del13	Positive
9	F	†22	APS-1	A	-	21OH, 17OH, TH	c.769C>T/c.1242_1243insA	Positive
10	M	49	APS-1	A, C	-	21OH, SCC, GAD	c.967-979del13/c.967-979del13	Positive
11	F	28	APS-1	A, C, HP	E	21OH, 17OH, SCC, NALP5	c.769C>T/c.1242_1243insA	Positive
12	F	-	AAD	-	-	21OH	No	Negative

**A, adrenocortical failure; Al, alopecia; HP, hypoparathyroidism; C, candidiasis; E, enamel hypoplasia; V, vitiligo; G, hypogonadism; K, keratoconjunctivitis; N, nail hypertropia; M, malabsorption; 21OH, 21-hydroxylase; 17OH, 17-hydroxylase; NALP5, NACHT leucine-rich-repeat-protein 5; SSC, side-chain cleavage enzyme; GAD, glutamic acid decarboxylase

†, deceased (age in years)

-, not found

8.2. Appendix II

RNA expression profiling of genes related to immune tolerance and T cell anergy – data for all genes. Positive fold change indicates higher expression in patient than in control, a negative fold change on the other hand indicates a lower expression in patient than in control. A p-value under 0.01 is considered statistically significant.

Gene	Fold change	P-value	Comment
BTLA	1.08	0.671	B
CBLB	1.28	0.139	Okay
CCL3L1	2.39	0.219	A
CCR4	1.77	0.298	C
CD27	1.42	0.798	B
CD28	-1.51	0.853	B
CD40	1.37	0.555	Okay
CD40LG	-1.68	0.413	B
CD70	1.12	0.252	Okay
CDK2	-1.07	0.739	Okay
CDK4	1.31	0.987	Okay
CMA1	1.34	0.948	B
CSF1	1.24	0.603	Okay
CSF2	1.49	0.323	A
CTLA4	-1.45	0.488	C
DGKA	-1.08	0.722	Okay
DGKZ	1.02	0.739	Okay
EGR2	-1.00	0.484	B
EGR3	1.19	0.307	B
EOMES	-1.31	0.678	Okay
FAS	1.11	0.686	Okay
FASLG	-5.06	0.0396	Okay
FOS	-1.55	0.269	Okay
FOXP1	-1.10	0.575	Okay
FOXP2	-1.44	0.394	B
FOXP3	-1.12	0.505	Okay
GATA3	-2.24	0.333	Okay
GZMB	-1.12	0.937	B
HDAC9	1.81	0.276	Okay
ICAM1	-1.09	0.941	A
ICOS	26.91	-	B
IFNG	3.44	0.475	B
IL10	6.69	-	B
IL10RA	9.62	-	B

IL13	-3.71	0.434	B
IL15	1.70	0.430	Okay
IL17A	-	-	C
IL1A	-1.13	0.199	Okay
IL2	-1.27	0.924	B
IL2RA	159.78	-	A
IL2RB	1.97	0.395	C
IL31	-1.06	0.586	C
IL4	1.40	0.321	B
IL5	-1.21	0.943	B
IL6	1.21	0.144	Okay
IL7R	-3.81	0.840	Okay
ING4	1.04	0.825	Okay
IRF4	-1.70	0.926	B
ITCH	-1.02	0.883	Okay
ITGA1	1.21	0.345	Okay
JAK1	1.43	0.915	Okay
JAK3	4.70	0.337	B
JUN	-4.87	0.559	Okay
LAT	1.41	0.336	Okay
LEP	1.01	0.385	B
LGALS3	-2.55	0.496	Okay
LTA	4.95	0.337	B
MEF2A	-1.22	0.417	Okay
NFATC1	-1.27	0.979	Okay
NFATC2	2.14	0.176	C
NFATC3	-1.05	0.457	Okay
NFKB1	1.58	0.0524	Okay
NHLH2	2.52	0.494	C
NOTCH1	-1.17	0.847	Okay
PDCD1	2.70	0.459	C
PRF1	-1.12	0.673	B
PRKCG	-1.01	0.975	B
PTGER2	-1.12	0.407	Okay
PTGS2	-1.26	0.257	B
RNF128	-	-	C
SELL	-1.16	0.776	B
STAT3	-2.59	0.564	Okay
STAT6	-1.00	0.851	Okay
TBX21	12.16	0.263	B
TGFB1	1.04	0.929	Okay
TNFRSF10A	-1.39	0.872	Okay
TNFRSF14	1.05	0.888	Okay

TNFRSF18	-1.02	0.519	B
TNFRSF4	1.09	0.505	C
TNFRSF8	-1.07	0.494	C
TNFRSF9	1.19	0.257	B
TNFSF10	1.59	0.298	B
TNFSF14	-2.30	0.223	C
TNFSF8	5.06	0.172	B
ACTB	-	-	Okay
B2M	1.11	0.565	Okay
GAPDH	-1.31	-	Okay
HPRT1	-1.00	0.934	Okay
RPLP0	-	-	Okay

*** A – High threshold level in either patient or control and low in the other.

B – High threshold level, low expression in both patient and control. P-value > 0.05.

C – Average threshold not determined or greater than the cut-off value in both patient and control. Expression not detected and fold-change result are erroneous.

-, no fold change/p-value obtained.

8.3. Appendix III

Number of cells obtained from samples used in isolation of PBMC. Five were chosen as controls in cell culture, flow cytometry, qPCR and ELISA assays (marked blue).

Number	Sex	Year of birth	Cell count x 10^6
62	M	1985	0.500
63	M	1970	0.100
73	M	1978	0.270
74	M	1965	0.380
75	M	1969	0.497
76	M	1978	0.619
80	F	1997	2.85
83	M	1990	8.30
84	M	1971	4.80
85	F	1992	8.20
88	M	1980	1.71
89	M	1998	2.33
90	F	1981	2.93
91	F	1999	1.12
92	M	1960	4.52
93	F	1983	3.92
94	M	1963	1.24
95	M	1968	1.76
96	M	1950	2.83
97	M	1953	3.54
98	M	1992	2.98
99	F	1971	4.42
100	F	1978	3.01
101	M	1975	1.71
147	M	1966	5-10
149	M	1967	5-10
208	M	1992	3.47

8.4. Appendix IV

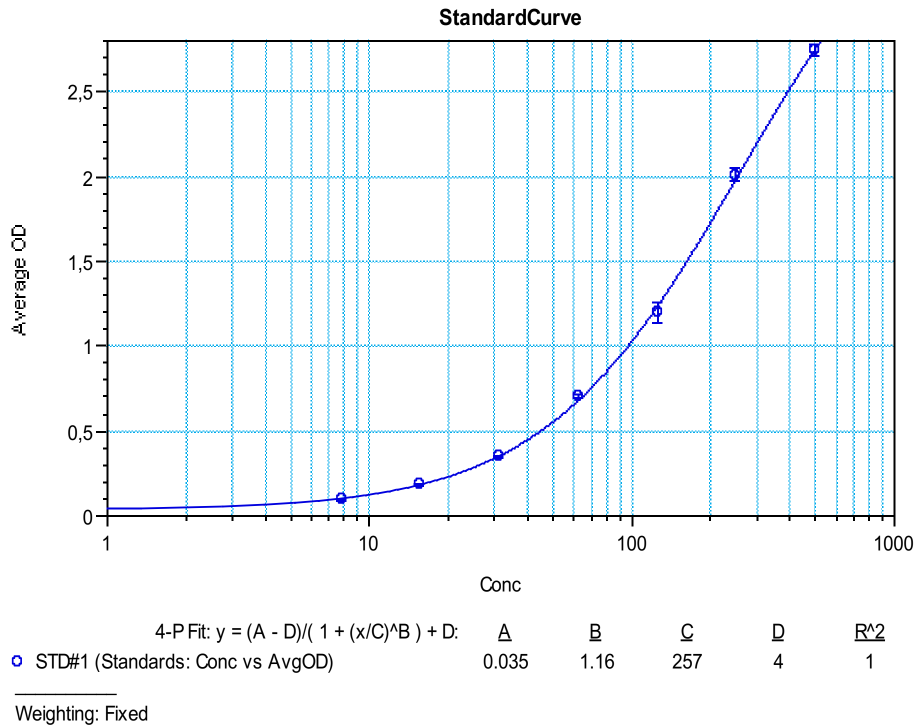
Obtained RNA concentrations and RIN values for patients and controls 1-5.

	RNA concentration [ng/μL]				RIN values			
	No drug patient	**Drug patient**	**No drug control**	**Drug control**	**No drug patient**	**Drug patient**	**No drug control**	**Drug control**
1	11.62	6.56	56.69	2.58	6.30	6.70	4.60	-
2	39.60	61.83	13.72	5.23	5.90	6.80	6.70	6.70
3	4.36	0.85	4.10	1.07	8.0	-	7.70	4.60
4	4.11	7.71	4.34	4.56	-	-	6.60	5.80
5	5.42	1.33	6.22	3.35	6.60	5.60	9.10	9.20

**** -, no RIN values obtained due to low sample volume.

8.5. Appendix V

Standard curve from one of the performed ELISA assays.



Appendix V figure 1: ELISA standard curve. *An example of a standard curve taken from one of the ELISA assays performed. The curve is made based on declining concentrations of a standard solution, including a blank sample. For each assay a new standard is run.* R^2 *equals one, indicating a perfect standard curve. Concentrations of IFN-γ could be calculated from the curve, but in this case the software provides these values directly. Samples that fall outside the range of the standard curve most likely need to be reanalyzed at a higher or lower concentration.*