From Department of Medicine, Huddinge
Karolinska Institutet, Stockholm, Sweden

EXPLORING CANNABINOIDS FOR INDOLENT B-CELL LYMPHOMAS

Christopher Melén

Karolinska Institutet

Stockholm 2024

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2024

ISBN 978-91-8017-347-6
Cover illustration: Hoburgen, Gotland 2015

Exploring cannabinoids for indolent B-cell lymphomas

Thesis for Doctoral Degree (Ph.D.)

By

Christopher Melén

The thesis will be defended in public at Jan-Åke Gustafson, Blickagången 16 Huddinge, 2024-05-17 09.00

Principal Supervisor:
Associate Professor Björn Wahlin
Karolinska Institutet
Department of Medicine, Huddinge
Division of Hematology

Co-supervisor:
Professor Birgitta Sander
Karolinska Institutet
Department of Laboratory Medicine
Division of Pathology

Opponent:
Associate Professor Kristina Drott
Lund University
Department of Laboratory Medicine
Division of Hematology and Transfusion Medicine

Examination Board:
Associate Professor Jeanette Lundin
Karolinska Institutet
Department of Oncology-Pathology

Professor Sören Lehmann
Uppsala University
Department of Medical Sciences
Division of Haematology

Professor Christer Sundström
Uppsala University
Department of Immunology, Genetics and Pathology

To Louise, Liv and Karin

”Ingenting får hända dig
Nej vad säger jag
Allt måste hända dig
och det måste vara underbart”

-Bodil Malmsten

POPULAR SCIENCE SUMMARY OF THE THESIS

The immune system, or, simplified, “the white blood cells” normally protects us from infections and it also tries to kill defect and cancerous cells in our bodies. The immune system is made up by several different cell types, with various tasks and specializations. The B lymphocyte (or B cell) is one of the white blood cells. Normally it lies waiting within a lymph node for other white blood cells to come and show evidence of an ongoing infection. It then starts to multiply and adjust itself according to what infection is present so it can start producing antibodies which in turn can help the immune system defeat the infection.

Sometimes the B lymphocyte itself becomes damaged and when that happens the cell can start to ignore the normal rules of behaviour and instead multiply uncontrollably. If this continues, a cancer can form and multiply and it is then called a lymphoma. Because cells of the healthy immune system normally travel via blood, cancers that arise from these cells are often already spread throughout the body when discovered. In fact, several types of lymphomas are almost always present in the blood. These diseases are among those that were named “leukaemia” when discovered.

Lymphomas that originate from the B lymphocytes are often treatable even when the disease is spread to several parts of the body. If the lymphoma is fast growing then intravenous chemotherapy can be effective enough to cure patients without the need to operate and remove the cancer. If the lymphoma is slow growing or “indolent”, then treatment as we know it today can not cure the patients. Instead, it pushes the lymphoma back (the patient attains “remission”) so we can not see it anymore and the patients can then often go back to living their lives as normal for 5 or even 10 years before the disease starts growing again. When this happens, the disease is often treatable again with new therapies. The problem is that the treatments often contain chemotherapy which have side effects and can lead to problems with for example infections and fatigue and that, finally the patients will run out of available treatments.

Therefore, doctors and researchers are constantly looking for new and less toxic ways to treat lymphomas. One such possible treatment are the chemicals that are found in the cannabis-plant. These chemicals, or “cannabinoids” as they are called, are known to have several different effects in humans and have been used for both medical and recreational purposes for a long time in human

history. My group has previously investigated what effects cannabinoids have on two types of lymphomas which often overexpress the genes for the cannabinoid receptors. We saw that lab-grown cells that mimic the behaviour of mantle cell lymphoma (MCL) died when exposed to high concentrations of cannabinoids and also that when we put live lymphoma cells into mice, their tumors shrank after we gave them cannabinoids.

For my PhD-project, I have recruited patients with slow growing lymphomas which have spread to the blood. They have then been given a mouth-spray containing cannabinoids. We could see that lymphoma cells in the blood clearly decreased in number after the treatment, but we could not see anything that indicated that the lymphoma cells had died. Instead, we think that the cannabinoids made the cancer cells move away from the blood into other parts of the body. We also investigated how long after their administration, the cannabinoids were detectable in the patients by analysing saliva, breath, blood and urine. The most interesting finding here was that even a small dose of cannabinoids could be detected in the patient's urine also one week after treatment. Our next studies tried to explain what cannabinoids do to the lymphoma cells by analysing how the cells move in an experimental setting as well as looking at how the cells change their expression of genes. In those studies, we could see that cannabinoids do affect lymphoma cell migration and that genes connected to migration and proliferation seemed to be activated more after cells were exposed to cannabinoids.

In conclusion, we could not see any evidence that cannabinoids could be used to kill lymphoma cells in humans. One likely possibility is that we couldn't get to the high doses needed to kill the lab-grown cells since the side effects of the cannabinoids started to be too severe. Our suggestion is that cannabinoids should not be used when treating lymphomas since the lymphoma cells are likely harder to kill when they have left the blood stream.

ABSTRACT

The overexpression of cannabinoid receptors is well described in several indolent lymphomas but the relevance of this finding is more uncertain. Our group has previously shown that mantle cell lymphoma (MCL)-derived cell lines known to overexpress the cannabinoid receptor 1 entered apoptosis after exposure to cannabinoids and that lymphomas xenografted onto mice shrank in size after exposure to cannabinoids. To further understand the potential effects of cannabinoids on indolent lymphomas, we therefore undertook a clinical trial and subsequent correlational studies which are described in this thesis.

In the trial, we recruited 23 patients with leukemic indolent B-cell lymphomas from our hematologic out-patient clinic. The patients were given cannabinoids in the form of a mouth-spray (Sativex®) with doses escalating between the patients to identify the maximal tolerated dose. The patients were blood sampled at regular intervals, both during a control day and on the day on the cannabinoid study drug administration. After a week, a final sample was taken. This longitudinal sampling allowed us to investigate what, if any, effects the cannabinoids had on the indolent B-cell lymphoma cells. Our analysis told us that the absolute number of circulating lymphocytes decreased after administration of cannabinoids but there was no evidence of apoptosis, neither was there any evidence of decreased proliferation, in the lymphoma cells. Instead, we surmised that a migration of lymphocytes away from the blood stream was the most likely explanation to our findings.

The second study focused on the pharmacological perspectives of the above-described clinical trial. All 23 patients underwent testing of captured breath, saliva, urine and blood during their participation in the study. From this we learned that the newer method of captured breath to detect cannabinoids was very sensitive and gave false positives if not very carefully handled. We also found that the urine analysis was surprisingly sensitive for a long period of time and in some cases cannabinoids were detected a week after administration in patients that received very small doses of the study drug. Both these findings have practical implications, for example when testing is conducted in workplaces and in traffic monitoring.

Our third study investigated the possible interplay between the two types of cannabinoid receptors and how they affect cell migration. We used primary cells from patients and several different MCL-cell lines to discern what type of

cannabinoid receptor was important in cell migration. We also investigated how the receptors communicate with each other and interact with the known potent chemokine receptor C-X-C Chemokine Receptor type 4 (CXCR-4).

Paper IV is a manuscript detailing our ongoing work to identify the effects cannabinoids have on lymphoma cells. In this paper we take frozen lymphoma cells from the clinical trial and analyse them using RNA-sequencing (RNA-seq). Since we had cells saved from all the various timepoint of the study, we could produce a longitudinal analysis of how mRNA-levels changed after administration of cannabinoids.

LIST OF SCIENTIFIC PAPERS

I. **Christopher M. Melén***, Magali Merrien*, Agata M. Wasik, Georgios Panagiotidis, Olof Beck, Kristina Sonnevi, Henna-Riikka Junlén, Birger Christensson, Birgitta Sander** & Björn Engelbrekt Wahlin**. Clinical effects of a single dose of cannabinoids to patients with chronic lymphocytic leukemia *Leuk Lymphoma.* 2022;63(6):1387-1397.

II. **Christopher M. Melén**, Magali Merrien, Agata M Wasik, Birgitta Sander, Björn Engelbrekt Wahlin, Georgios Panagiotidis, Olof Beck. Δ9-THC and CBD in Plasma, Oral Fluid, Exhaled Breath, and Urine from 23 Patients Administered Sativex. *Cannabis Cannabinoid Res* 2023 Apr 19 Online ahead of print.

III. Magali Merrien, Agata M. Wasik , **Christopher M. Melén**, Mohammad Hamdy Abdelrazak Morsy, Kristina Sonnevi, Henna-Riikka Junlén, Birger Christensson, Björn E. Wahlin and Birgitta Sander. 2-Arachidonoylglycerol Modulates CXCL12-Mediated Chemotaxis in Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia. *Cancers*. 2023 Mar; 15(5): 1585.

IV. **Christopher M. Melén**, Magali Merrien, Agata M. Wasik, Birger Christensson, Birgitta Sander & Björn Engelbrekt Wahlin. Longitudinal RNA-seq on indolent lymphoma cells before and after exposure of cannabinoids in vivo. Manuscript 2024

* first authors equal contribrution
** last authors equal contribution

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
5HT1a	Serotonin 1A Receptor
ABHD6	Alpha/Beta Domain Containing Hydrolase 6
ABHD12	Alpha/Beta Domain Containing Hydrolase 12
AE	Adverse event
AEA	N-arachidonoyl-ethanolamine
AKT	Protein kinase B
ATF	Aktivating Transcription Factor
BCL2	B-cell lymphoma 2
BTK	Bruton's Tyrosine Kinase
CB1	Cannabinoid Receptor-1
CB2	Cannabinoid Receptor-2
CBC	Cannabichromene
CBD	Cannabidiol
CBG	Cannabigerol
CCR7	C-C chemokine receptor 7
CCL21	C-C motif chemokine ligand 21
CD	Cluster of Differentiation
CHOP	Cyclophosphamide, doxorubicin, vincristine sulfate, prednisone
CHOP	C/EBP homologous protein
CINV	Chemotherapy-induced nausea and vomiting
CLL	Chronic Lymphocytic Leukemia
cMCL	classical Mantle cell lymphoma
CMG	Chromatin modifying genes
CRF	Case report forms
CNS	Central Nervous System
COX-2	Cyclooxygenase-2

CXCR4	C-X-C chemokine receptor type 4
CXCR5	C-X-C chemokine receptor type 5
CXCL12	C-X-C motif chemokine ligand 12
CXCL13	C-X-C motif chemokine ligand 13
Δ9-THC	Δ9-tetrahydrocannabinol
ECS	Endocannabinoid system
EGF	Epidermal growth factor
EMA	European Medicines Agency
ERK	Extracellular signal-regulated kinase
FAAH	Fatty Acid Aminohydrolase
FDA	Food and Drug Administration
FL	Follicular Lymhoma
GI	Gastrointestinal
GPCR	G Protein-Coupled Receptor
GPR18	G Protein-Coupled Receptor 18
MAPK	Mitogen-Activated Protein Kinase
MCL	Mantle Cell Lymphoma
MGL	Monoacylglycerol Lipase
MS	Multiple sclerosis
MZL	Marginal Zone Lymhoma
NAPE	N-arachidonoyl Phosphatidyl Ethanol
NFAT	Nuclear factor of activated T-cells
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
nnMCL	non-nodal leukemic Mantle cell lymphoma
PPARγ	Peroxisome Proliferator–Activated Receptor γ
PI3K	Phosphoinositide 3-kinase
THCV	Tetrahydrocannabivarin
TRB3	*tribbles*-related protein 3

TRP	Transient Receptor Potential
TRPM	Transient Receptor Potential subclass M
TRPV	Transient Receptor Potential subclass V

INTRODUCTON

Indolent B-cell lymphomas are hematological disease entities which are considered uncurable with the treatments available today (1). Most of the patients with a newly discovered indolent lymphoma does not need treatment at diagnosis (1). Instead, the disease it typically monitored through what is called "wait-and-watch" and then treated if and when the disease starts to cause symptoms such as weight loss, night sweats, fevers, and/or growth of lymph nodes which can compress and impair surrounding organs such as blood vessels and for example kidneys (2).

Treatment for indolent B-cell lymphomas has traditionally been a combination of antibodies, such as the CD20-targeting monoclonal antibody Rituximab, and chemotherapy, such as bendamustin, or the cyclophosphamide, doxorubicin, vincristine sulfate, prednisone (CHOP)-regimen (3). Typically, the indolent B-cell lymphoma is treated to remission and the patient then returns to the wait-and-watch monitoring (4).

There are however complications associated with both the disease itself and with the treatments which lead to an excess mortality in patients with indolent B-cell lymphomas compared to a control population. Typically, the period of time a patient is in remission between treatments decreases for each time treatment is administered, leading to a scenario where we have no more tolerable treatments to offer (5).

There has been a rapid development of new treatments the last 10 years with new, more targeted, therapies being available to patients. In 2013 the novel drug ibrutinib was approved in the US, followed by approval in the EU in 2014 (6). This drug is a Bruton's tyrosine kinas (BTK)-inhibitor and it is taken continuously and typically has milder side effects compared to the traditional antibody + chemotherapy treatment options. BTK inhibitors were followed by other targeted therapies such as phosphoinositide 3-kinase (PI3K)-inhibitors, B-cell lymphoma 2 (BCL-2)-inhibitors and new generations of BTK-inhibitors (7–9).

Still, the underlying problem persists and most of the patients with indolent B-cell lymphomas will progress through treatments if given enough time. Therefore, there is a continuous search for new therapeutic options with tolerable toxicity for patients with indolent B-cell lymphomas.

Cannabinoids have been suggested to have antineoplastic effects in lymphomas and other malignancies. Our group have previously shown that cannabinoids can induce apoptosis in MCL cell lines and to decrease the proliferation of malignant cells and shrink lymphomas xenografted onto mice. In this thesis we continue to investigate the effects of cannabinoids in indolent B-cell lymphomas.

1 LITERATURE REVIEW

1.1 A BRIEF HISTORY OF CANNABINOIDS

The use of *Cannabis* has been recorded throughout human history. Archaeologists have detected remnants of *Cannabis sativa* in Chinese agricultural societies dating back 8000 years, using the fibres of the plant for rope manufacture but the seeds and leaves for nutrition and possibly medicinal uses (10). In Assyria 800 B.C., *Cannabis sativa* had two names, one when used as medicine against epileptic seizures (azaullu) and one when recreationally used (gan-zi-gun-nu, which translates to "the drug that takes away the mind") (11). Both words trace their origin to the much older Sumerian language and civilization. The knowledge of *Cannabis sativa* as a recreational drug and possible medical substance was carried on in India, Persia and throughout mediaeval Arabic cultures (12). The first modern description of the medicinal qualities of the plant was done in 1839 when the Irish physician O'Shaughnessy showed the effect of hemp extracts on tetanus and rabies, two infectious diseases in which the extract of *Cannabis indica* was supposed to lessen the symptoms of muscular rigidity and spasticity (13).

In the 1960s several groups identified the active compounds in the plant and a whole group of chemical compounds was described and given the name "cannabinoids". Δ9-tetrahydrocannabinol (Δ^9-THC) was found to be the only cannabinoid to induce all four symptoms of the tetrad of clinical features associated with the use of *Cannabis sativa* in mice – catalepsy, hypokinesia, hypothermia and antinociception – and was therefore recognised as the principal active agent (14). In 1988 the cannabinoid receptor-1 (CB1) was discovered (15) and in 1992 an endogenous ligand, N-arachidonoyl-ethanolamine (AEA or anandamide) was found (16) and further investigations have led to the description of a whole cannabinoid system of various receptors and ligands.

2. THE ENDOCANNABINOID SYSTEM (ECS)

2.1 CANNABINOID RECEPTOR 1 AND 2

After the discovery of CB1 (encoded by the gene *CNR1*), the cannabinoidreceptor-2 (CB2) (encoded by the gene *CNR2*) was characterised in 1993 (17). CB2 was predominantly found on immune cells in peripheral blood and in lymphatic tissue such as the marginal zone of the spleen and the Peyer's patches of the gastrointestinal tract whereas CB1 initially was found solely in neurons in the CNS. This led to a model of a cannabinoid system which was anatomically divided into a "central" and a "peripheral" part. This paradigm lasted for a decade until there was sufficient evidence that there is a considerate overlap between the locations of CB1 and CB2. It is now known that also CB2 is present in the CNS and that CB1 is found in physiologically relevant levels in cells in organs such as the liver, skeletal muscles, adipose tissue and the pancreas (18).

Both receptors belong to the rhodopsin subfamily of G protein coupled receptors (GPCRs) and share the ability to influence several different signalling pathways when active. The main effect seen when CB1 and/or CB2 is activated is inhibition of adenyl cyclase and activation of mitogen-activated protein kinase (MAPK) cascades. CB1 activation alone has also been shown to modulate Ca2+-channels and to activate channels which facilitate an influx of K+ into cells (19). All GPCRs share certain characteristics. They are defined by their structure: seven transmembrane helical domains linked together by loops and with an extra-cellular N-terminus and intra-cellular C-terminus (20). Canonically, a GPCR is activated by its ligand which leads to conformation changes at its intracellular domains and subsequent recruitment of heterotrimeric G protein complexes. The G protein complex then undergoes dissociation and the various sub-units activates other pathways and thus induce effects in the target cell. After activation a GPCR is marked by β-arrestin (21). This often initiates internalization of the receptor and the GPCR can then be either degraded or recycled back to the cell surface. This allows the cells to regulate the number of available receptors and thus also the continued response to stimulation. β-arrestin has also been shown to initiate its own downstream signalling apart from simply initializing internalization. This function, in which one ligand to one receptor can initiate two separate downstream signals is called "biased signalling" (22,23). It has perhaps been best investigated in the opioid- and dopamine- GPCRs (24–26). For the opioid-receptor there is evidence that that the sought after analgetic effects are induced by the G protein sub-unit signalling and that unwanted side effects such as constipation and respiratory depression instead

depend on the biased signalling from β-arrestin (27). In 2021 the US Food and Drug Administration (FDA) approved olicerdine, a synthetic ligand for the opioid-receptor (28). Ligation of olicerdine to its receptor results in recruitment of β-arrestin in only 14% of events, meaning that fewer biased signals are initiated and much fewer receptors are internalized. In studies the substance has shown better efficacy than standard morphine and possibly have lesser adverse events and might represent a shift in how GPCRs can be targeted with drugs (29,30).

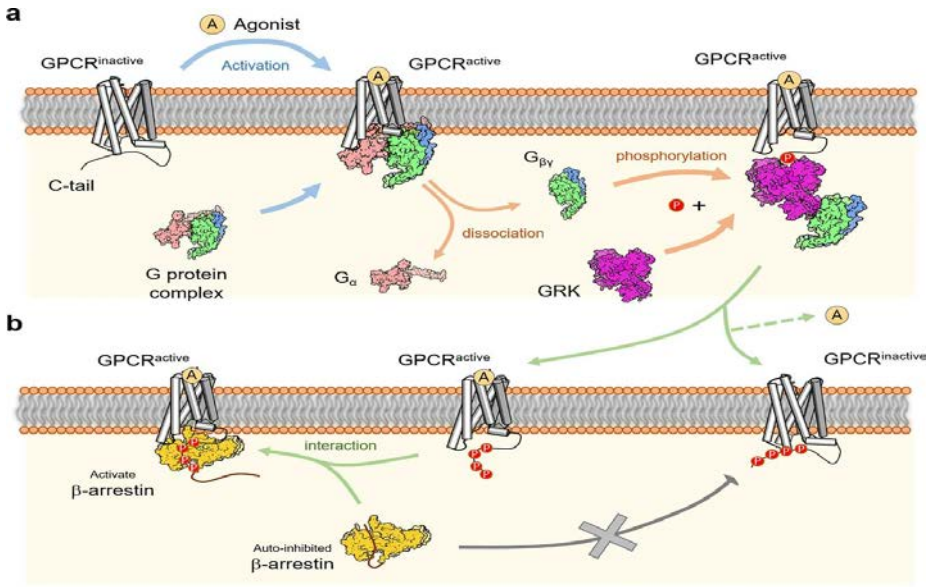

Figure 1 Schematic illustration of the activating procedure of GPCR as well as the agonist-induced signalling event. **a** (Up) The heterotrimeric G protein complex interacts with the activated GPCRs, therefore triggers the dissociation of the Gα and Gβγ subunit, where the latter recruits GRKs to phosphorylate the C-terminal tail of GPCR. **b** (Down) The β-arrestin tends to preferentially interact with activated and phosphorylated GPCRs than the phosphorylation-only GPCRs. (reprinted under the Creative Commons Attribution 4.0 International License from Xu Z and Shao Z. Dynamic mechanism of GPCR-mediated β-arrestin: a potential therapeutic agent discovery of biased drugs *Signal Transduct Target Ther*. 2022; 7; 283)

GPCRs are capable of functioning as monomers but that they often form other structures, either with another GPCR of the same type (homodimerization) or with another type of GPCR (heterodimerization) (31–33). Higher forms of structures are also described such as tetramers and various oligomers (34). GPCR-heterodimers are the most extensively studied of these various forms and results show both synergistic and inhibitory downstream signalling depending on which GPCRs make up the heterodimer and also that formation of various heterodimers can influence how GPCRs get internalized and recycled (35–38). A brief overview of possible results from GPCR-heterodimerization is presented in Figure 2.

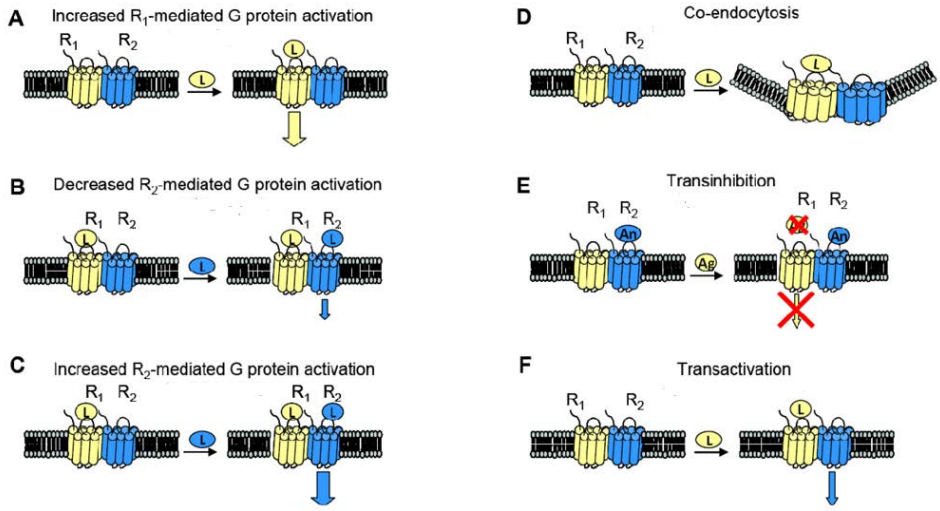

Figure 2. Functional consequences of GPCR heterodimerization. (A). Co-expression of a second receptor can lead to increased G protein activation by the stimulated receptor; (B). In the presence of agonists of both receptors, there can be dampened response to a receptor's cognate ligand in the presence of a second receptor as compared to when each receptor is expressed singly; (**C**). Conversely, the addition of a second receptor can increase G protein activation in response to a receptor's cognate ligand; (**D**). Heterodimerization with a second receptor can also change receptor trafficking with stimulation of either R1 or R2 leading to co-endocytosis of both receptors; (**E**). Addition of an antagonist against one of the two receptors prevents the other receptor from binding agonist and/or activating, leading to trans-inhibition; (F). Alternatively, one receptor's ligand can lead to transactivation of the second receptor's signalling pathway as opposed to activating R1's cognate pathway. (adapted with permission from Haack K and McCarty N. Functional Consequences of GPCR Heterodimerization: GPCRs as Allosteric Modulators *Pharmaceuticals*. 2011; 4(3), 503-53)

Recent studies have also demonstrated that the localisation of CB2 can vary in healthy B-cells and that CB2 is no longer detectable on the cell surface on B-cells with activated IgD- (39). Interestingly, CB2 is then still exerting effect on the cell from intracellular niches which might represent yet another layer of modulation of CB2 signalling in that B-cells could then prevent CB2-containing GPCR heterodimers from forming on the cell surface while still retaining signalling through CB2 since the lipophilic ligands can transverse the cell membrane.

2.2 LIGANDS FOR THE CANNABINOID RECEPTORS

With over 15 endogenous ligands for CB1 and/or CB2 found and with a wide variety of synthetic cannabinoids being constructed, a nomenclature has been adopted. “Endocannabinoids” are endogenously produced substances that have effects mainly through CB1/CB2. Exclusivity is not a criterion; an endocannabinoid can induce activity in other receptors than CB1/CB2 and thus AEA was not only the first endocannabinoid found but also the first endogenous

ligand to the Transient receptor potential (TRP) subfamily V (40,41). "Phytocannabinoids" are defined as compounds found within plants from the *Cannabaceae* family that exhibit effects on CB1/CB2, either directly as a ligand or indirectly by affecting the concentrations of the endocannabinoids by inhibition/activation of enzymes responsible for their production and/or degradation. "Synthetic cannabinoids" constitute the third group of relevant compounds: substances designed and produced for specific effects on CB1/CB2 or on the enzymes important for the production or degradation of endocannabinoids.

Going through the full list of ligands for the cannabinoid receptors is beyond the scope of this thesis. Instead, we here present the most relevant endo- and phytocannabinoids. Δ^9-THC acts as a partial agonist with high affinity to both CB1 and CB2 but is far from the only compound in *Cannabis sativa* with the ability to bind to these receptors. Cannabidiol (CBD) was discovered in 1963 (42). It shows low affinity to CB1 and thus it does not induce the typical tetrad of symptoms in mice described above. CBD has instead been shown to act as a weak antagonist on CB1 in mice (43) and as a weak inverse agonist on CB2 (18), meaning that it binds to CB2 and but instead of activating it, CBD turns off some of the continuous activity of the CB2 receptor. CBD also acts as an "indirect agonist" on CB1 and CB2 by inhibiting hydrolysis and removal of AEA (19) and of 2-arachidonoylglycerol (2-AG). 2-AG is considered the most abundant endogenous cannabinoid and is a full agonistic ligand to CB1 and CB2 (44). The second most common endocannabinoid is AEA which functions as a partial agonist to CB1 and CB2 (45). There are over 120 known compounds that qualify as phytocannabinoids (46) but few of these have clinically recorded relevant effects. There is however evidence that the various phytocannabinoids interacts when whole plant *Cannabis sativa* is used and, just to mention a few, cannabigerol (CBG), cannabichromene (CBC) and tetrahydrocannabivarin (THCV) have been shown to act as ligands on CB1 and/or CB2 and also to exert influence on the ECS by inhibition of the endocannabinoid-degrading enzyme fatty acid aminohydrolase (FAAH) (47–49) in a similar fashion as CBD.

2.3 PRODUCTION AND DEGRADATION OF CANNABINOIDS

Studies of the production, transportation and degradation of endocannabinoids have mostly been performed on AEA and 2-AG. They are described as produced "on demand" and not continuously produced and/or stored in vesicles (50). However, some studies have suggested that some storage capabilities might exist in microglial cells which could use extracellular membrane vesicles called "exosomes" both as storage and as a mean to transport the strongly hydrophobic endocannabinoids over the intracellular space in the synaptic gap (51).

AEA is mainly produced from N-arachidonoyl phosphatidyl ethanol (NAPE) and 2-AG is produced from phospholipids containing 2-arachidonoyl (52–54). 2-AG is also, in itself, an intermediate in the synthesis of lipids and is especially important for the production of prostaglandins where it is a major source of arachidonic acid. Any intervention in the production of 2-AG is highly likely to affect production of prostaglandins which have key functions in a wide variety of organs such as kidneys, liver, lungs, heart and the gastrointestinal (GI)-tract (55).

The degradation of AEA and 2-AG are also mainly separate but has some possible interplay. AEA is mainly degraded by FAAH and, to a lesser extent by, cyclooxygenase-2 (COX-2) whereas 2-AG is predominantly degraded by the hydrolytic enzymes alpha/beta domain containing hydrolase 6 (ABHD6), alpha/beta domain containing hydrolase 12 (ABHD12) and monoacylglycerol lipase (MGL). Under certain conditions, 2-AG can also be oxidized by COX-2 and then further hydrolyzed by FAAH and thus interact with the degradation of AEA (56).

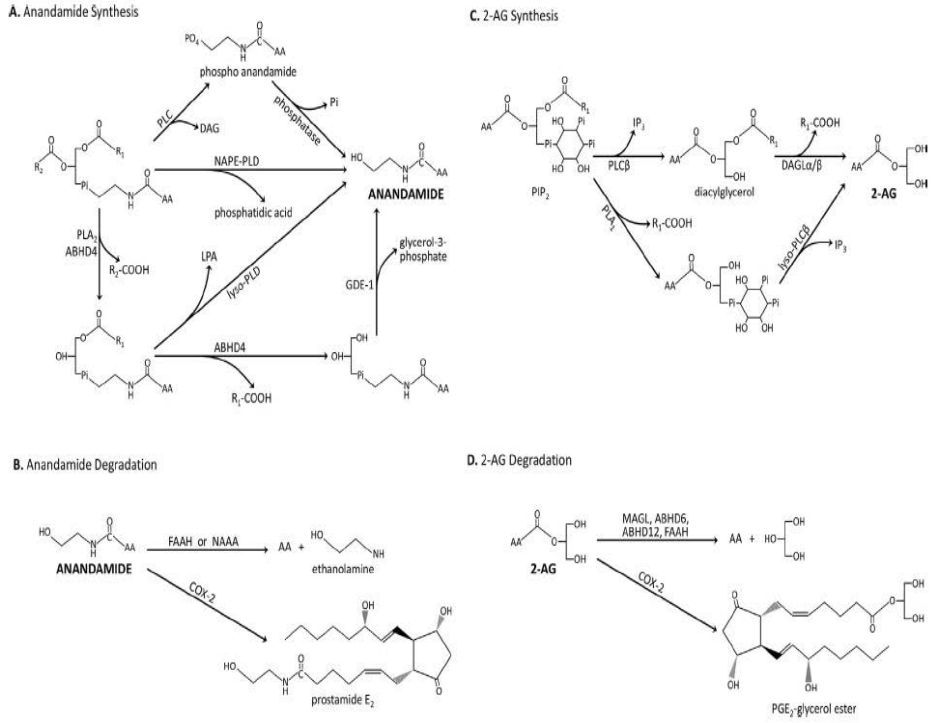

Figure 3 Potential synthetic and degradative pathways for anandamide and 2-arachidonoyl glycerol (2-AG). (A) Primary synthetic pathways for anandamide. (B) Primary degradative pathways for anandamide. (C) Primary synthetic pathways for 2-AG. (D) Primary degradative pathways for 2-AG. Only major pathways are shown. AA, arachidonic acid; ABHD4, alpha/beta domain containing hydrolase 4; ABHD6, alpha/beta domain-containing hydrolase 6; ABHD12, alpha/beta domain-containing hydrolase 12; COX-2, cyclooxygenase-2; DAG, diacylglycerol; DAGL, diacylglycerol lipase; FAAH, fatty acid aminohydrolase; GDE-1, glycerophosphodiester phosphodiesterase I; IP3, inositol trisphosphate; LPA, lyso-phosphatidic acid; lyso-PLC, lyso-phospholipid-preferring phospholipase C; MAGL, monoacyl glycerol lipase; NAAA, N-acyl ethanolamine amino hydrolase; NAPE-PLD, N-arachidonoyl phosphatidyl ethanol-preferring phospholipase D; PIP2, phosphatidyl inositol bis-phosphate; Pi, PO4; PLA2, phospholipase A2; PLC, phospholipase C. (reprinted under Fair Use Protocol from Lu H and Mackie K. An Introduction to the Endogenous Cannabinoid System *Biol Psychiatry*. 2016; Apr 1; 79(7): 516–525.)

2.4 TARGETS OUTSIDE ECS

CBD has also demonstrated effects on several receptors not classically designated as cannabinoid receptors. This includes several GPCRs, which are classified as "orphan receptors", such as GPR18 (partial agonist) (57) and GPR55 (antagonist) (58–62). CBD also binds to receptors of the transient receptor potential family (TRP), especially on the vanilloid (TRPV) subfamily (63–65). Furthermore, CBD acts as a positive allosteric modulator on glycine receptors (66), activates PPARγ-receptors (67) and has a slight affinity for the $5HT_{1a}$-

receptor (68). CBD is not the only compound in *Cannabis sativa* to have such a multi-target effect. Δ^9-THC has also been shown to exert influence on other receptors than CB1/CB2, with the allosteric modulation of the $5HT_{3a}$-receptor being the most clinically relevant finding (69). Apart from these two constituents the other phytocannabinoids found in *Cannabis s.* also demonstrate various direct/indirect effects on receptors outside of ECS with several acting as ligands on TRP- and PPARγ-receptors (46).

3. CLINICAL ASPECTS OF CANNABINOIDS

3.1 APPETITE, METABOLIC SYNDROME AND EMESIS

In 1992 FDA approved dronabinol, a synthetic cannabinoid analogue of Δ^9-THC, for use against HIV/AIDS-associated weight loss (70). Dronabinol has also been used against chemotherapy-induced nausea and vomiting (CINV) and weight loss associated with CINV or malignancy (71,72). Effects on CINV are likely due to dual activity on CB1 and $5HT_{3a}$ but the use is limited by general activation of CB1 in the CNS and subsequent psychoactive effects. CBD has shown antiemetic properties in animal models, acting through activation of $5HT_{1a}$-receptors in the brainstem but there are no well-designed studies in humans.

In 2005 the European Medicines Agency (EMA) approved rimonabant for treatment of obesity. Rimonabant is an CB1 antagonist and was tested in a clinical study with weight loss as main end point (73). The study showed that administration of 5mg or 20mg of rimonabant in combination with diet and exercise resulted in significantly greater weight loss than diet and exercise alone with 3.4kg and 6.6kg of weight loss respectively for the rimonabant-arms as compared to 1.8kg of weight loss with diet and training alone. Adverse events reported in the study was mostly mild but follow-up data showed that over 30% of patients using rimonabant developed psychiatric adverse events such as depression and anxiety, likely due to inhibition of CB1 in the CNS but also with likely off-target effects on other GPCRs in the CNS such as dopamine (74). This eventually led to the discontinuation of the drug in 2009 (75). Several companies are developing monoclonal antibodies with CB1-agonisitic properties (76). Furthest along is nimacimab which is starting a phase 2-trial 2024. This antibody is described as a negative allosteric-modulator of CB1 with tolerable side effects and does not pass over the blood-brain-barrier and should thus not

induce the same psychiatric adverse events as rimonabant (77). The intended purpose of the antibody is not weight loss but regulation of the metabolic syndrome with hopes to improve the cardiac-, kidney and liver function in patients with obesity and poor metabolic control. Rimonabant has also been refurbished in a nanoparticle capsule form in an effort to utilize CB1-modulation without the risk of CNS-mediated adverse events (Figure 4) (78). The rationale for this approach is that the nanoparticle capsule will break down in the liver and deposit the drug locally and thus with fewer CNS-mediated side effects.

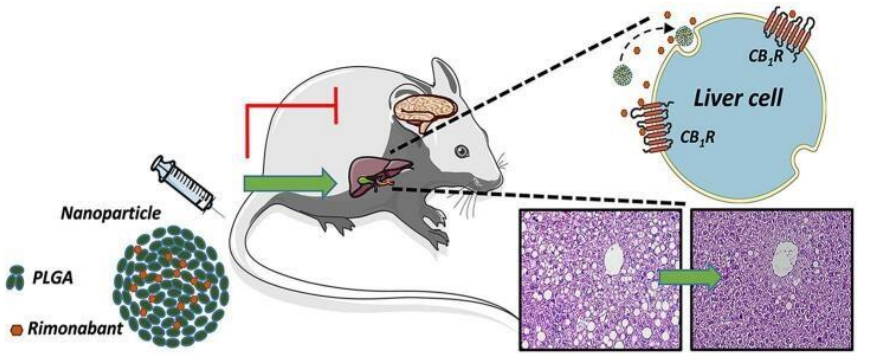

Figure 4 A Summary of the concept with administration of Rimonabant in capsulated form. Arrow in green indicates local effect in target organ (liver) without effect in CNS. Pictures bottom right indicates decreased severity of fatty liver. (printed with permission from Hirsch S *et al*. Hepatic targeting of the centrally active cannabinoid 1 receptor (CB1R) blocker rimonabant via PLGA nanoparticles for treating fatty liver disease and diabetes *J Control Release*. 2023 Jan; 353: 254–269.)

3.2 PAIN, ANTI-INFLAMMATION AND GLAUCOMA

Δ^9-THC modulates acute and chronic pain in animal models. Signalling through CB1 alone seems to be important for modulation of acute pain (78) whereas modulation of chronic pain, of either neuropathic or inflammatory origin, requires activation of both CB1 and CB2 (79,80). CBD has the capacity to modulate neuropathic and inflammatory pain in rodents through indirect effects on CB1/CB2 via inhibition of AEA degradation and modulation of the TRPV1-receptor (81). Both CBD and Δ^9-THC modulate inflammatory pain through anti-inflammatory actions in animal models such as altering the production of the chemokines such as TNF-α, IL-1β, IL-6, IL-12 and IL-10 (82). Δ^9-THC also affects

the expression of adhesion molecules, affecting migration, proliferation and apoptosis of immunological cells engaged in inflammatory responses (83).

In 2016, a phase I study investigating the potential use of an FAAH-inhibitor was terminated prematurely as one patient died and 5 others were hospitalized due to serious adverse events (84). The rational for this study was that inhibition of FAAH would lead to increased and persisting concentrations of endocannabinoids since they would not be degraded. This in turn would lead to analgetic effects. The serious adverse events that manifested were headaches, hemiparesis, memory impairment, loss of consciousness and diplopia. On magnetic resonance imaging anomalies were seen which indicated vascular bleeding in hippocampus, pons, thalamus and the cerebral cortex. After further analyses, no direct cause for the adverse events could be found and other FAAH-inhibitors that were being tested in studies showed no similar adverse events. Neither of these did however show efficacy and no FAAH-inhibitor is available as a registered drug today.

The effect of *Cannabis sativa* on glaucoma is well investigated and it is known since the 1970s that inhalation or ingestion of the plant can decrease the intraorbital pressure associated with the condition by relaxation of the trabecular meshwork (85–87). More recent studies have also demonstrated potential neuroprotective properties facilitated by cannabinoid receptors in the retina due to inhibition of the production of nitric oxide (88,89). This could potentially decrease the neurodegeneration in the retina which is otherwise a potential result of long-term glaucoma. A locally administered ophthalmic emulsion with the synthetic CB1 agonist SBI-100 finished enrolment for its phase 2 study in February 2024 but results are at the time of printing of this thesis not yet published (90).

3.3 NEUROLOGICAL AND PSYCHIATRIC DISEASES

CBD and Δ^9-THC have been investigated for their anticonvulsant properties. Treatment with Δ^9-THC alone has a variety of dose-dependent effects in animals, including a rebound hyperexcitability leading to increased sensibility for new convulsions. It is thus unsuitable as a therapeutic drug for humans (91,92). CBD has been shown, both in *in vitro* and *in vivo*-models, to have anti-epileptiform and anticonvulsant qualities by regulating Ca^{2+} and through

activation/inactivation of TRPV1, TRPV2 and GPR55 (93–95). The FDA has approved CBD for the treatment of two rare congenital forms of epilepsy, Dravet and Lennox-Gastaut, after showing effect in several randomised controlled trials; the product is also approved by EMA to be used together with standard antiepileptic drugs (96,97). Δ^9-THC has well-known psychoactive properties through its activation of CB1 and has been studied several times in an effort to understand its potential role in schizophrenia (98). Use of Δ^9-THC can induce symptoms of psychosis in healthy individuals depending on dose, much as any intoxicating substance. In individuals with a genetic predisposition for schizophrenia, Δ^9-THC might induce an earlier onset of the disorder, and also aggravate its course. CBD has been tested to treat psychosis based on its properties as a weak antagonist of CB1 with demonstrated effects in small clinical trials (99,100). When given prior to the intake of Δ^9-THC, CBD alleviates some of the risk for acute psychotic symptoms, congruent with its activities on CB1 (101). In mouse models of multiple sclerosis (MS), Δ^9-THC decreases and CBD aggravates symptoms of spasticity, indicating a clear role of CB1/CB2 activation (102). In the same models, Δ^9-THC has been shown to lessen CNS inflammation and prolong survival (102). The oral Δ^9-THC/CBD-drug Sativex® was approved to treat MS related pain and spasticity in USA and EU after showing a significant reduction in spasticity with tolerable adverse events (103). This drug is a *C. sativa* whole-plant extract with a fixed amount of 2.7mg Δ^9-THC and 2.5mg CBD in each actuation. Single-agent treatment with Δ^9-THC has also been tried but did not show any significant delay of progression of symptoms in a well-constructed phase III-study spanning over 3 years when compared with placebo, except in a small sub-group of patients with a more aggressive variant of MS (104).

3.4 ANTINEOPLASTIC EFFECTS

CBD has shown antiproliferative and apoptotic properties in breast, colorectal, prostate carcinoma cell lines (105). When exposed to 10 µM CBD, activation of the intrinsic apoptosis pathway could be detected in prostate carcinoma cell lines (106). Furthermore, an increased production of radical oxygen species with known apoptotic features could also partly explain these apoptotic qualities as this could be seen in several cell lines and in an animal model of glioblastomas (105,107,108)). Inhibition of PPARγ, COX-2, TRPV1, TRPM8 or CB2 reversed the

anti-proliferative effects of CBD *in vitro* (109) and also in mice xenografted with human lung carcinoma cells, highlighting the PPARγ-receptors potential role in this process (109). CBD in concentrations between 6 and 10.6 μM suppresses metastasis and cancer cell infiltration in a cell line used as model for metastasizing breast carcinoma (106,109,110), partly through inhibition of epidermal growth factor (EGF), NF-κβ, ERK/AKT, and matrix metalloproteinase 2 and 9 pathways (111). CBD in levels ranging from 1 to 9 μM reduces angiogenesis in vitro and in mice xenografted with human umbilical vein endothelial cells (112).

Δ^9-THC in concentrations 3-20 μM inhibits growth of several malignant cell lines *in vitro* and in xenografted mice-models, corresponding to doses of ≥3 mg/kg (113–116). The anti-proliferative and apoptotic effects of Δ^9-THC vary between the cell lines but several studies have indicated that increased production of pro-apoptotic sphingolipid ceramides is important (114). In glioma cell lines a ceramide-dependent upregulation of the stress protein p8 resulted in the upregulation of the endoplasmic reticulum stress-related genes ATF-4, TRB3 and CHOP and in increased autophagy-mediated apoptosis via activation of the mTORC1 complex axis (117). Similar mTORC1 activation with autophagy-mediated apoptosis was also seen in hepatocellular carcinoma cell lines (118). In a cell line derived from acute T lymphoblastic leukaemia, elevated ceramide levels were detected after the administration of a synthetic Δ^9-THC-analogue, which led to downregulation of the Raf-1/MEK/ERK/RSK pathway, resulting in activation of pro-apoptotic BAD (119). Activation of p38 MAPK could be seen in another study of the same cell line with a resulting increased apoptotic activity (120). In cell lines representing colorectal cancer inhibition of RAS-MAPK/ERK and PI3K-AKT activates BAD, leading to increased apoptosis (121).

Through in situ proximity ligation assays, which can identify physical closeness of proteins *in vitro*, it has been shown that CB2 can form heterodimers with GPR55, which has known tumour-promoting functions (122). The heterodimers are active and influence intracellular cAMP-levels and signalling through the p-ERK-1/2 pathways. The heterodimers are activated by low concentrations of Δ^9-THC through CB2 but as concentrations of Δ^9-THC rise it starts to act as an antagonist to the heterodimers, likely through activity on GPR55. How common such heterodimers are in normal biological processes are not well investigated but functional CB1/GPR55-hetromers have been found in the striatum of the CNS

in both rat and monkeys (123) and active CB1/CB2-heterodimers have been detected in several parts in the CNS of rats (124)) indicating that the ability to form heterodimers could be common for CB1 and CB2.

There are no real epidemiologic data on whether cannabinoid use decreases or increases risk for malignancy (125). Cannabis smoke has shown carcinogenic effects in bacterial models and in mice but it has not been shown to lead to an increase of cancer in human cohorts (125,126). No prevention of cancer development has been shown from cannabinoid use.

4. B-CELL MALIGNANCIES

B-cells can give rise to a multitude of different types of lymphomas. This is explained by the several different steps of development the benign B-cell undergoes during its lifetime and the major development stages are described here in Figure 5.

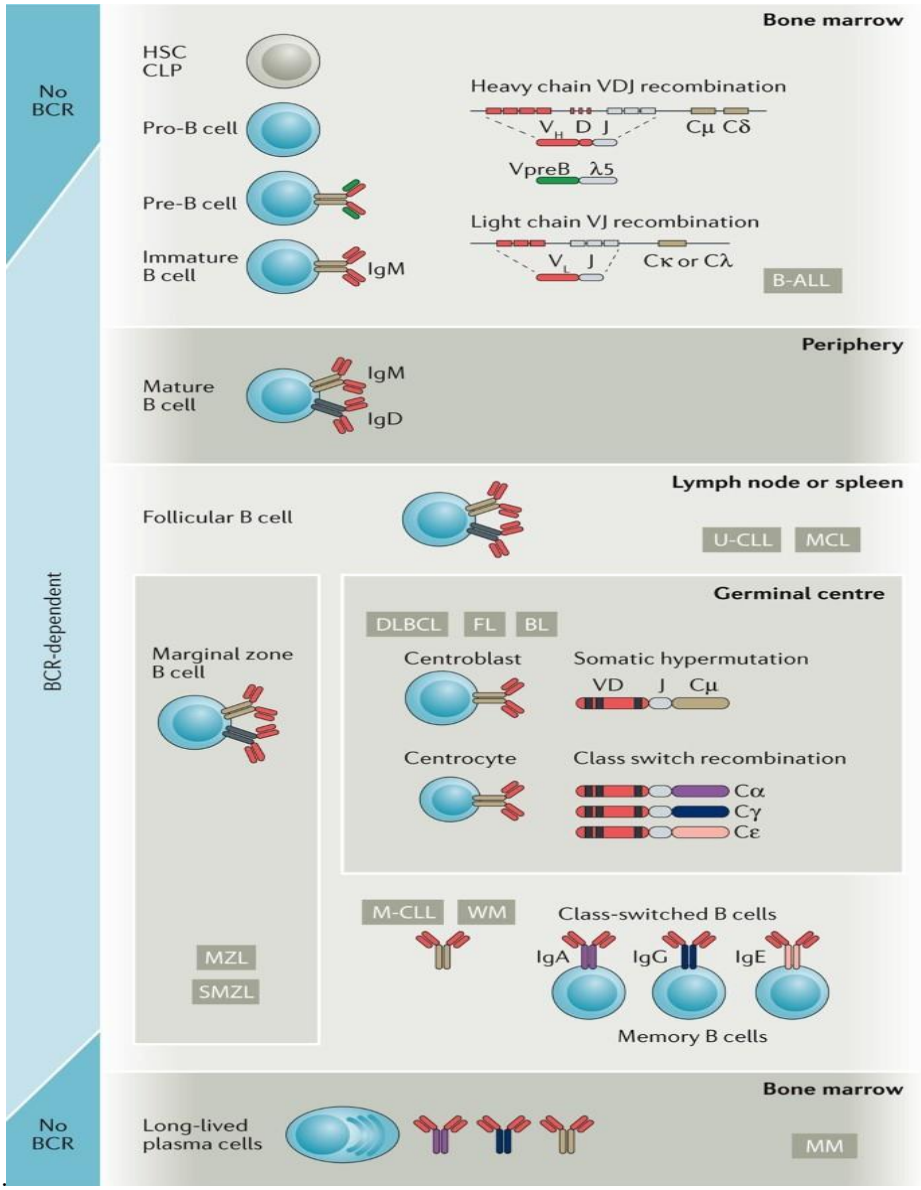

Figure 5 Illustration of major steps in a B-cells development and a selection of B-cell lymphomas that can arise from the different stages of development. Abbreviations: BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; HSC, haematopoietic stem cell; MCL, mantle cell lymphoma; M-CLL, Immunoglobulin gene mutated chronic lymphocytic leukaemia; MM, multiple myeloma; MZL, marginal zone lymphoma; SMZL, splenic marginal zone lymphoma; U-CLL, Immunoglobulin gene unmutated chronic lymphocytic leukaemia; WM, Waldenstrom macroglobulinaemia. (adapted with permission from Burger, J.A. & Wiestner, A. (2018) Targeting B cell receptor signalling in cancer: preclinical and clinical advances. Nat Rev Cancer, 18, 148-167)

Translocations are genetic insults which are potentially potent oncogenic drivers and these are more likely to occur as the more immature B-cells undergo heavy- and light chain recombination in the bone marrow as compared to in a cell undergoing normal mitosis (127). Similarly, as the more mature B-cells undergo somatic hypermutation of immunoglobulin genes (and other genes as a bystander effect) and immunoglobulin class switch in the germinal centre, there is also an increased possibility for genetic alterations leading to lymphoma development (128).

Homing to different tissues during different stages of the B-cells' life is a key feature for normal B-cell development. Malignant B-cells can dysregulate chemokines and adhesion molecules to allow the cell to stay in a certain microenvironment in which it receives proliferative and antiapoptotic signals. It has been shown that the cells in several leukemic B-cell malignancies upregulate and/or auto-activate the B-cell receptor (129,130). The lymph node microenvironment promotes NF-κβ signalling in malignant B-cells (131,132). Key receptors involved in the homing to tissues are the chemokine receptors C-X-C chemokine receptor type 4 (CXCR4), C-X-C chemokine receptor type 5 (CXCR5) and C-C chemokine receptor 7 (CCR7). These receptors are part of the same GPCR-superfamily of receptors as the cannabinoid receptors and have specific ligands to activate them under healthy circumstances – the chemokine C-X-C motif chemokine ligand 12 (CXCL12) activates CXCR4 to home the B-cells to the bone marrow, C-X-C motif chemokine ligand 13 (CXCL13 acts on CXCR5 for positioning cells in the germinal centre of the lymph node and C-C motif chemokine ligand 21 (CCL21) activates CCR7 to allow B-cells to enter lymph nodes (133).

4.1.1 CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most common indolent lymphoma type and has a variable clinical course (133). CLL is classically associated with four chromosomic abnormalities that all leads to increased cell survival. Loss of the important tumour suppressor *TP53* by deletion of the short arm of chromosome 17 (del 17p) causes an aggressive form of CLL which responds poorer to standard antibody + chemotherapy (134,135) whereas deletion of the long arm of chromosome 11 (del 11q) and subsequent loss of the *ATM* gene involved in DNA damage detection leads to a disease with a generally shorter

time to first treatment from diagnosis but with a more favourable response to standard treatment when the two are compared (134,136). Deletion of the long arm of chromosome 13 (del 13q) leads to loss of *BCL2* silencing factors and subsequent upregulation of the anti-apoptotic B-cell lymphoma 2 (BCL2) protein and has the most favourable general outcome of the known aberrations (134,137). Trisomy of chromosome 12 (+12) correlates to several distinct clinical features such as non-leukemic manifestation of the disease, increased risk of autoimmune complications and an increased risk of Richter transformation, in which part of the CLL gains additional mutations and behave as an aggressive lymphoma (138). The exact mechanics as to how +12 can lead to these various phenotypes are not fully discovered but could be in part driven by activation of the Bruton's tyrosine kinase (BTK) through aberrant activity in the nuclear factor of activated T-cells (NFAT) pathway and by overexpression of the immune checkpoint molecule NT5E (CD73).

4.1.2 FOLLICULAR LYMPHOMA

Follicular Lymphoma (FL) is a disease arising from germinal centre B-cells. A key event for FL is the chromosomal translocation t(14;18)(q32;q21)*IGH::BCL2* which is found in 70-90% of all FL patients. With this translocation, the encoding gene for BCL2 is moved to the vicinity of the *IGH* enhancer, leading to an overexpression of BCL2 and subsequent anti-apoptotic features (139). This aberration alone is not enough for FL to develop as shown by studies in which as many as 53% of healthy test subjects was found to carry B-cells with the translocation (140). The t(14;18) takes place during the V(D)J heavy chain recombination of the pro-B-cell and is believed to create a cell that mimics a normal B-cell in its development until it reaches the germinal centre. There, the t(14;18)-carrying B-cell are less likely to initiate apoptosis after antigenic selection due to the overexpression of BCL2. This results in an accumulation of t(14;18)-carrying B-cell which can accrue further mutations, progressing them into FL (141,142). Mutations to chromatin modifying genes (CMG) is observed in around 90% of FL and likely benefit the lymphoma through epigenetic changes which allow the cells to remain in the germinal centre instead of being forced to exit it or differentiate to a memory B-cell or plasma cell (143). This is important for FL since it relies heavily on signals from the germinal centre microenvironment for proliferation and it has been shown that the composition of the microenvironment predicts prognosis (144).

4.1.3 MANTLE CELL LYMPHOMA

Mantle cell lymphoma (MCL) is a disease which is typically described as a semi-aggressive lymphoma, with rapid onset of symptoms and often in need of treatment at diagnosis (145). MCL shares with indolent lymphomas the inability to be completely cured with available treatments, with the exception of the rare cases when the patient presents with localised disease and curative radiotherapy can be administered. The development of MCL starts with a translocation of t(11;14)(q13;q32)*CCND1::IGH* during the pre-B-cell stage when the cell undergoes V(D)J heavy chain recombination (139). This translocation leads to overexpression of the cell cycle promoting protein cyclin D1 since its encoding gene, *CCND1*, is moved to be under the influence of the *IGH* enhancer. This enhancement of proliferation is not enough for the development of MCL and the translocation itself can be found in circulating lymphocytes in healthy individuals without signs of MCL (146,147). Additional mutations, most often in genes involved in cell cycle control, DNA repair and/or cell survival must occur and thus mutated *BCL2*, *TP53*, *CDK4* and/or *RB1* is often seen in MCL (148–151). MCL can be further divided into classical MCL (cMCL) and non-nodal leukemic MCL (nnMCL) (152). cMCL typically presents with an unmutated *IGHV* gene and is believed to not have entered the germinal centre and thus have not undergone somatic hypermutation of *IGHV*. These cells originally reside in the mantle zone of the lymph node but can also grow diffusely and give rise to the classical symptoms of an aggressive lymphoma with enlarged lymph nodes and rapid onset of symptoms. nnMCL was initially thought to be an atypical CLL due to its leukemic presentation and mutated *IGHV* but as it also carries t(11;14) it was later classified as MCL (153). nnMCL is clinically characterized by a more indolent progression of disease in that the patients do not need to undergo treatment until burden of disease starts to grow. This correlates well with that nnMCL generally carries fewer additional mutations as compared to cMCL (154,155).

4.1.5 MARGINAL-ZONE LYMPHOMA

The marginal-zone lymphomas (MZL) represent an entity of indolent lymphomas that are particularly associated with underlying chronic inflammation and/or infection. MZL are mainly divided into three separate types: the splenic MZL

(sMZL) and nodal MZL (nMZL), which are often associated with systemic inflammations such as the ones caused by chronic hepatitis C, and extra nodal MZL of mucosa associated lymphoid tissue (MALT lymphoma). MALT lymphomas in turn are driven more by more organ specific infections/inflammations (156).

The development of gastric MALT lymphomas is the best studied pathogenesis of the various MZLs and is often used as a model for all MZL development (157). The classic driver for gastric MALT is chronic infection by *Helicobacter p.* which generate an immune response that preferentially expand B-cell clones with B-cell receptors reactive to the pathogen. This clone is embedded in the supportive immune microenvironment but, as the infections fails to clear and becomes chronic, the sustained proliferation signals can lead to deregulation of pathways which can then be aggravated by acquired molecular lesions and eventually leading to a tumour which is no longer reliant on the microenvironment for support. There are distinct genetic features associated with the various underlying triggers, and thus with the various MZLs, but the molecular lesions tend to result in dysregulated NF-κB, B-cell receptor and/or NOTCH (158–161).

Clinically, NMZL and SMZL are often systemically spread when diagnosed (157). As with the other indolent lymphomas, MZLs are only treated if symptomatic. The various MALT lymphomas are more often diagnosed due to symptoms such as gastric pain and/or reflux but en passant-findings are also common (162,163). The finding of an M-component of IgM-type can result in the diagnosis of a MZL since 30% of patient with MZL have an M-component producing disease (164).

5. CANNABINOIDS AS POTENTIAL DRUGS FOR INDOLENT LYMPHOMAS

Some evidence supports the hypothesis that cannabinoids have antineoplastic activity especially in hematologic malignancies. In MCL, it has been shown that the genes coding for CB1 and CB2, *CNR1* and *CNR2*, are overexpressed in nearly all patients (165) and that this overexpression might be clinically relevant because low expression of *CNR1* correlates with lymphocytosis and indolent disease (166). Accordingly, cMCL is associated with higher levels of *CNR1*-overexpression as compared to nnMCL (167). Furthermore, dysregulations in the

production and degradation of endocannabinoids have also been detected in samples from MCL patients. NAPE-PLD, a main enzyme in the production of both AEA and 2-AG is upregulated in nearly all investigated MCL-patients and the main degrading enzyme FAAH downregulated, suggesting elevated levels of endocannabinoids in tissues (166). Approximately 50% of investigated CLL samples overexpressed *CNR1* and *CNR2* compared to healthy B-cells and the CLL patients who overexpressed *CNR1* had shorter treatment-free and overall survival (116,168). When stimulated with endogenous-, synthetic- or phytocannabinoids, MCL cell-lines go into apoptosis (116,169) and/or paraptosis, a programmed cell death which shares several features with necrosis (170). When cells from an *CNR1* and *CNR2*-overexpressing MCL cell line were xenografted into mice and treated with a synthetic AEA-analogue, this led to reduced cell proliferation and approximately 40% smaller tumour (116). When the same synthetic cannabinoid was tested on cell lines from other lymphomas that overexpress only either *CNR1* or *CNR2*, no induced cell death could be seen, but when tested on cell lines overexpressing both genes, cell death could again be detected. This indicates that cannabinoids might induce cell death and that it appears to be dependent on both CB1 and CB2.

2 RESEARCH AIMS

Our main goal with this project was to explore the clinical effects of cannabinoids on the indolent B-cell lymphomas that are known to overexpress the cannabinoid receptors. We therefore conducted our clinical trial which is the first study of its kind in humans. As we planned for the study, we also wanted to investigate potential explanations for our clinical findings as well as further the knowledge of cannabinoids pharmacokinetic properties.

Specific aims for the studies:

I. Do cannabinoids induce significant changes in circulating malignant lymphocytes without inducing intolerable adverse events?
II. Are the non-invasive methods used to detect THC and CBD effective and safe to use? For how long can THC be detected in urine in previously cannabis-naïve patients?
III. How does the endocannabinoid 2-AG influence chemotaxis of benign and malignant lymphocytes and what, if any, are the interactions with CXCL12?
IV. Can the results seen in Paper I be explained by longitudinal RNA-sequencing of the lymphoma cells?

3 MATERIALS AND METHODS

3.1 CANNABINOIDSTUDIEN-1

In order to answer our questions, we initiated a prospective non-randomized, open label clinical study using the, in US and EU, registered drug Sativex. As described in section "3.3 NEUROLOGICAL AND PSYCHIATRIC DISEASES" of the literature review, this drug is registered to treat spasticity in MS and is an oral spray of whole plant *Cannabis s.* The spray dispenses 100 microliter per actuation corresponding to a fixed dose of 2.7mg Δ^9-THC and 2.5mg CBD per actuation. We received ethical permission from the proper authority. We also applied to the Swedish Medical Products Agency (Läkemedelsverket). We applied because we aimed to give a drug with known psychotropic side-effects outside of its registered use, and we wanted our study to undergo as extensive review beforehand as possible. After setting up a formal clinical trial protocol including case report forms (CRFs), dose escalation program, grading of adverse events (AEs) and informed consents, we received permission to start our study (Dnr 5.1-2015-91825). We also registered the study in EU Clinical Trials Registry (EudraCT 2014-005553-39).

Initially, the patients were enrolled the same day they came to the out-patient clinic to receive the study drug at 9:00 AM and then stayed during the work day to be tested repeatedly and monitored for adverse events. They returned to the out-patient clinic the morning after and the week after for continued sampling and were then done in the study (Figure 6).

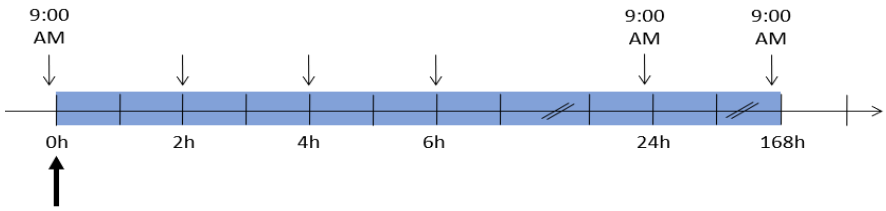

Figure 6 Illustration of a patient's participation in the Cannabinoidstudien-1. "9:00 AM" indicates that the patient arrives to the out-patient clinic and also that the patient leaves a urine sample. Thin arrow above the line indicates each time the patient undergoes sampling of blood, oral fluid and exhaled breath. Thick arrow below the line indicates when study drug is administered.

We had decided on a dose escalation based on registered AEs in pairs of patients in order to find the maximum tolerated dose. This meant that every two

patients received identical amount of Sativex, starting with one single actuation, and we then evaluated the adverse events.

If the adverse events were "mild or non-existing" and "non-severe" according to grading with the help of Common Terminology Criteria for Adverse Events (CTCAE) v4.0 the next two patients increased the dose with two more actuations. The same applied if the AEs had been "moderate" but still "non-severe" and if the investigator thought the AE was non-related. If the AE was "moderate" and "possibly related" or "related" to the study drug, then the next two patients stayed on the same dose and if the next two patients also had "moderate" and "possibly related" or "related" AEs, then the maximum tolerated dose was decided and no further dose escalation would take place.

We had a pre-planned interim analysis after our 10th patient had participated. We could at this time see an emerging pattern with decreasing circulating lymphocytes but with no evidence of apoptosis. We decided that we needed to expand the study with a control day to further sample our patients for a full day without any cannabinoid intervention and also to take an extra sample of the patients already one hour after administration of Sativex in order to try and see any rapidly induced changes. We paused the study to apply for these changes with both the Ethical board and the Medical Products Agency. After these changes were approved the study could restart and all patients thereafter undertook a full day of testing during a "control day" and then came back to the out-patient clinic a period of time later for the "intervention day" (Figure 7).

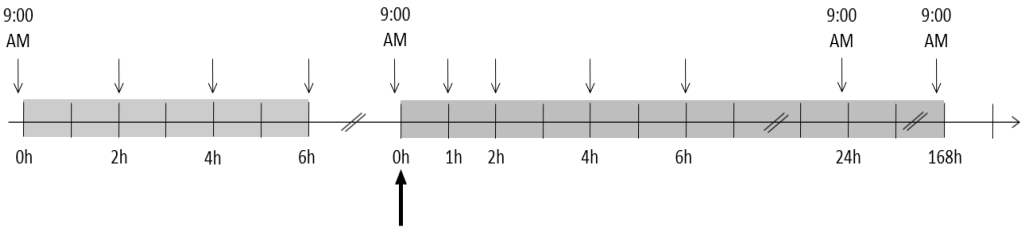

Figure 7 Illustration of a patient's participation in the Cannabinoidstudien-1 post interim analysis. Furthest to the left is the "control day" where the patient arrives at 9:00 AM and is sampled for blood at the time points of the thin arrows. "9:00 AM" after the broken timeline indicates when the patient arrives to the out-patient clinic to start the "intervention day" and also when the patient leaves a urine sample. Thin arrow above the line indicates each time the patient undergoes sampling of blood, oral fluid and exhaled breath. Thick arrow below the line indicates when study drug is administered.

3.1 ETHICAL CONSIDERATIONS

The major ethical issue regarding this thesis is that of the safety of the patients enrolled in our clinical intervention study. Since we could not assume that one administration of cannabinoids would induce any long-term beneficiary effects, we could not allow the patients to be put in a situation where they suffered potentially severe adverse events from the study drug. The patients who participated in the trial had known their doctors for a long time. They had thus likely built a relationship with a lot of trust involved in said clinicians. We had to take precautions to make sure that the patients joined the study out of their own free will and not a misguided sense of loyalty towards their treating clinician as well as to be careful with the doses of the cannabinoid study drug involved so no harm was done to the patients.

In order to resolve this issue, the patient's own clinician was not allowed to do the recruiting and enrolment of patients. Instead, another doctor involved in the trial would recruit and enrol. This led to a process in which we had a far from 100% success with the enrolment which at least indicates that patients joined or passed up on the study out of their own free will. Regarding the potential harm to the patients, we constructed a careful dose-finding part of the study with a slow increase of the dose between the patients included in the study. We also did set an absolute limit in that if a patient would suffer such a difficult adverse event so that he or she would need to be admitted to the hospital, then we would stop all further enrolment in the study. The informed consent used was approved by both the Ethics committee and the Swedish Medical Products Agency (Läkemedelsverket).

Ethical Permits:

Paper I, II and IV: 2015/281-31/2 with amendments 2016/210-32 and 2017/556-32

Paper III: 2003-12-08/689-03 with amendments 2009-1244-31-4, 2011/1053-31, 2014/48-32, 1222/12-631

I have met most of the 23 included patients on routine clinical follow-ups. I have then taken the opportunity to ask them if they would hypothetically join a new study with cannabinoids under the same premises as the first one. The response from the patients have been that of 50/50 between positive and negative attitudes towards rejoining a such a hypothetical follow-up study with the ones

having more pronounced adverse events naturally having a more negative attitude. No patient however expressed remorse for having joined the study.

3.2 PATIENTS AND PATIENT SAMPLES

We collected *in vivo* data as well as material for *ex vivo*-experiments. As the psychotropic adverse events became more frequent and persistent with increased dose of the study drug, the patients needed monitoring. This was managed by collaboration between the study nurse and the principal investigator or sub-investigator on duty so that the patient was never alone in the clinic.

Due to the prevalence of the diseases, we knew that most of our patients in paper I would have CLL. We had hoped to accrue at least two and hopefully three patients with MCL since this is a lymphoma that is known to have a frequent and strong over-expression of CB1 and was of special interest for us when we were setting up the study but we also decided that we should not try to select our patients but instead just enrol suitable patients as they showed up in the clinic. In the end, 20 of our patients included had CLL and only 3 had another kind of leukemic indolent lymphoma (1 MCL, 1 FL and 1 MZL) and whenever one of the 3 "non-CLL" patients had a divergent result, we could never know if it was because of individual properties of that patient or due to something present/missing due to their disease.

A key step for preparing patient samples of peripheral blood for our *ex vivo*-experiments was to purify the B cells. This was done through enrichment with antibodies (RosetteSep®) designed to target platelets and all leukocytes except B-lymphocytes. The targeted cells then adhere to erythrocytes and separate from the B-cells when centrifugated using Ficoll-Paque. This procedure left us with very high-purity B-cells of which the overwhelming majority were malignant (verified by flow cytometry) and represented the patient's disease. This step required two people to work simultaneously in the laboratory as new patient samples came in too frequently for one person to handle.

3.3 MCL CELL LINES AND PRIMARY CELLS

For paper III, we performed several experiments involving MCL cell lines. Cell lines are very convenient to use due to their ability to be grown and thus can be used to reproduce experiments is a predictable and plannable manner. But it should also be remembered that cell lines often diverge from their cell-of-origin in key ways and that the results from cell line experiments should be reproduced in primary cells if possible. In paper III we encountered a typical such issue as we moved between cell lines and primary cells in the migration experiment. We started with using cells from MCL cell lines to calibrate and get familiar with the method and could get reliable and reproducible data, but when we switched to using primary cells with the same settings, we got no real usable data. After reviewing the method and the experiment, we found that the cells from the cell line were substantially larger in diameter than the primary cells which affected their capability to migrate through the pores in the plates used in the chemotaxis assay and that using the same settings for both would never give comparable data.

Furthermore, there are no reliable cell lines for CLL. This is a major issue since most of our patients had CLL and thus cannot be adequately represented in cell line-experiments.

Many indolent lymphomas are also especially reliant on tumor microenvironment for their sustained growth and normal malignant behavior, and this cannot be reproduced in cell line experiments even though co-culturing cells from cell lines can partially alleviate that issue.

Despite these objections towards cell lines, they are still integral to furthering our understanding of have malignant diseases work and function if used correctly.

3.4 CHEMOTAXIS ASSAY

We used a standard Boyden chamber to asses chemotaxis (Figure 8)

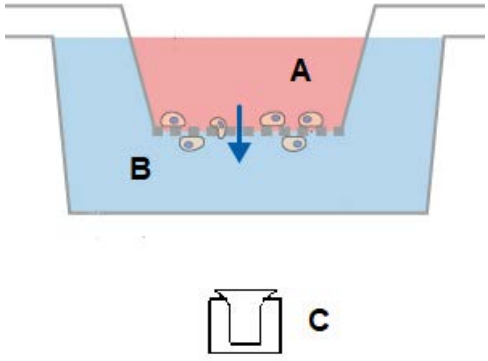

Figure 8 Schematic image of a chemotaxis experiment using a Boyden chamber. **A** Cells labelled with a fluorescent dye are inserted into the top. A porous membrane separates them from **B** which is a chamber containing the chemical to be investigated as a possible chemotactic inducer. **C** is a camera fixed under the chamber to allow read-out of migrated cells.

An important step to make to chemotaxis assay as reliable as possible was to ensure that the fluorescent staining was not released from the stained cells while incubating. Any release of staining would invariably lead to inconsistent and unreliable readings in the Boyden chamber.

The Boyden chamber itself needed to be fitted with different inserts for the pores since the cells from cell lines had an average size of 8 µm while the primary cells were on average 5 µm. We also needed to adjust the read-out from between the experiments. The primary cells and cells from the Jeko-1 cell line could be analyzed by standard measurement of number of fluorescent cells in the bottom chamber. But the cells from the Granta519 and JVM-2 cell-lines tend to form aggregates which make individual cell reading impossible. Instead, we opted to measure the intensity of the fluorescent activity as a surrogate marker when doing experiments with these cells.

3.5 FLOW CYTOMETRY

Flow cytometry on peripheral blood was performed using the pathology labs standard equipment. Flow cytometry was of great benefit to us in paper I since it gave us the possibility to assess ongoing apoptosis using the PhiPhiLux

fluorogenic protease substrate. This method allowed us to investigate if caspase 3 dependent apoptosis had been initiated in live cells and was our read out to determine cannabinoid-initiated cell death in the indolent lymphoma cells. Flow cytometry also allowed us to determine the surface expression of CXCR4 on the cells which is of key interest when investigating substances with chemotactic potential.

3.6 REAL-TIME PCR

There were no suitable antibodies for investigating the expression of cannabinoid receptors via flow cytometry, immunohistochemistry or western blot when we started working on paper I. Instead, we used rtPCR to quantify the expression of *CNR1* (coding for CB1) and *CNR2* (coding for CB2) as surrogates. To ensure relevant read-outs, we correlated the expression to the house-holding gene *ACTB* and also compared the expression of the study patients B-lymphocytes to that of B-lymphocytes from donated blood from healthy donors in order to determine "high" or "low" expression.

3.7 THC/CBD DETECTION

Oral fluid (OF) was obtained using the commercially available Quantisal collection device. With this system, the patient inserts a plastic spatula with a soft top buccally and waits until an indicator turns blue to ensure that the top is sufficiently saturated with OF. The entire device is then entered into a tube with a buffer solution and can then be frozen or instantly analyzed (Figure 9).

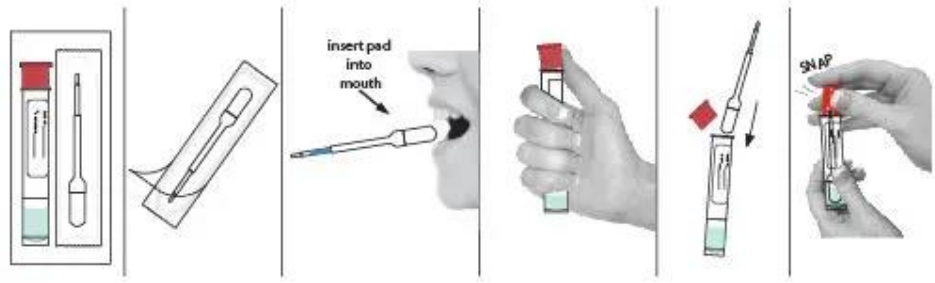

Figure 9 Schematic illustration of how to use the Quantisal collection device for oral fluid. (printed under Fair Use Protocol, copywrite Immunalysis Corp.)

We had issues with patients having dry mouth as an adverse event to their intake of cannabinoids. This led to failure to properly saturate the collections device, leading to failure to collect a sample all together from that timepoint as we had no back up planned for this eventuality even though it is a common side effect to use of cannabinoids.

Captured breath was obtained by the use of the SensAbues collection device (Figure 10). It is not the breath itself which is captured and analyzed. Instead, the device captures aerosol particles from alveolar cells, mostly consisting of fragmented alveolar cell membranes.

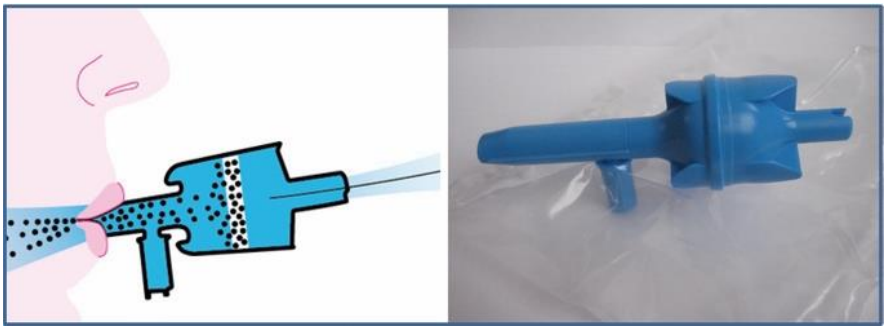

Figure 10 Schematic illustration of how to use the SensAbues collection device for captured breath (left) and real-life picture of the device (right). The mouthpiece is discarded and the housing containing the micro-particle filter is sealed and subjected for analytical investigation. (printed under Fair Use Protocol, copywrite SensAbues AB)

With this device we initially experienced several false positives, likely caused by contamination of the collection device by the research nurse as she handled the study drug. After stricter routines were in place, the device proved reliable.

The OF and captured breath was then frozen awaiting analysis using liquid chromatography coupled to a quadrupole tandem mass spectrometer (LC-MS/MS) as routine by our collaborators in the department of Clinical Pharmacology.

3.8 RNA-SEQUENCING

For paper IV, the mRNA was prepared by members of our group and delivered to the Bioinformatic and Expression Analysis (BEA) core facility in KI Huddinge. There the mRNA was sequenced on the Illumina platform using the stranded ligation assay (Figure 11).

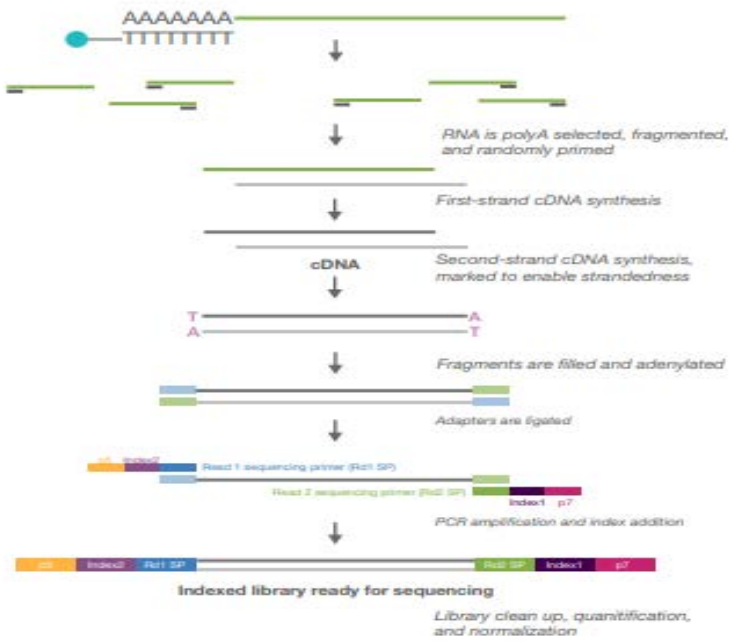

Figure 11 Illustration of the stranded ligation assay used for the RNA-seq. (printed with permission of Illumina Inc)

Reads were then mapped to the human genome using Spliced Transcripts Alignment to a Reference (STAR) and then the statistical computer program R was used with the DESeq2-package to normalize counts and identify differentially expressed genes.

3.9 STATISTICS

For paper I, II and III comparisons between repeated measurements were done, using the Wilcoxon matched-pairs signed-ranks test. Assessments of other associations were conducted using Fisher's exact, Spearman, or Mann–Whitney–Wilcoxon tests, according to the nature of the involved variables. p values are two-tailed and were calculated using Stata version 14.2 (StataCorp LLC, College Station, TX). For paper I, apoptosis and proliferation data were analyzed using GraphPad Prism version 8.3.0 (GraphPad software Inc., La Jolla, CA). $p < 0.05$ was considered significant. For paper II, Area under curve was calculated using Mann-Whitney U Test with Stata version 14.2 (StataCorp LLC, College Station, TX). For paper IV, Wards test was used to determine significance for the RNA-seq and was then adjusted for false discovery rate due to multiple testing correction

using the Benjamini and Hochberg-procedure. $p < 0.05$ was considered significant in combination with a log2-fold change of mRNA outside of -1 to 1.

4 RESULTS

Paper I: 23 patients were enrolled in the study, 20 with CLL and one each with FL, MCL and MZL. As expected, the cohort consisted mainly of elderly patients (median age of 73). The first two patients (patient 1 and patient 2) received 1 actuation of Sativex and reported just a mild AE of dry mouth. Thus, the dose was increased to 3 actuations. Again, the AEs were mild with one possibly unrelated AE. As the dose was then increased to 5 actuations, both patient 4 and 5 reported several AEs with a moderate euphoria in one patient. Following the trial protocol, the same dose of 5 actuation was given patient 6 and 7 . These two reported only mild AEs and the dose was again increased. At 7 actuations (corresponding to 18.9mg Δ^{9}-THC and 17.5mg CBD), we saw several moderate AEs in the next pair of patients (patient 8 and 9) and, per protocol, gave the same dose to the next two patients again (patient 10 and 11). As these two also reported moderate AEs, we had determined out maximum tolerated dose which was given to the remaining 12 patients. AEs continued to be noticeable but manageable during the study (Table 1).

Table 1 Adverse events.

	Grade 1	Grade 2	Grade 3-4	Total	Per cent
Dry mouth	18	0	0	18	78%
Vertigo	15	1	0	16	70%
Somnolence	7	3	0	10	43%
Hallucinations	7	0	0	7	30%
Confusion	2	2	0	4	17%
Euphoria	3	1	0	4	17%
Paresthesia	3	0	0	3	13%
Hypotension	0	2	0	2	9%
Rhinitis	1	0	0	1	4%
Hoarseness	1	0	0	1	4%
Nausea	1	0	0	1	4%
Vomiting	1	0	0	1	4%
Stomach pain	0	1	0	1	4%
Highest grade per patient	13	8	0	21	91%

Table 1 Summary of Adverse events of the 23 patients included. One patient could report several various AEs. (reprinted with permission from C. M. Melén *et al.* Clinical effects of a single dose of cannabinoids to patients with chronic lymphocytic leukemia *Leukemia & Lymphoma* (2022) Jun;63(6):1387-1397.)

The main finding in this paper is that there was a significant reduction in leukemic cells (median, 11%) which occurred within two hours after administration of the study drug ($p = 0.014$) (Figure 12). The effect remained for 6 h without evidence of apoptosis or change in proliferation. Patients with no overexpression of CB1 mRNA experienced a faster and more pronounced decrease as compared to the patients whose malignant cells overexpressed CB1 mRNA (Figure 13). Non-malignant B-cells and T-cells were also reduced. All effects were gone by 24 h. Secondly, we detected a possible diurnal rhythm of the circulating CLL-cells. This is not something that the study was set up to investigate but was detected during the longitudinal testing during the control day. We could also conclude that the study drug was tolerable to an elderly cohort even though psychotropic adverse events were common.

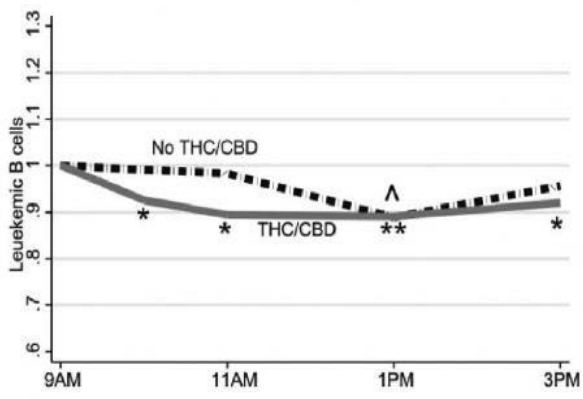

Figure 12 Changes in median levels of leukemic B-cells during the days without and with THC/CBD. For simplicity, only median values are shown here. One asterisk (*) and two asterisks (**) indicate significant changes at the day with treatment, with respect to baseline with $p < 0.05$ and $p < 0.005$, respectively. (reprinted with permission from C. M. Melén *et al.* Clinical effects of a single dose of cannabinoids to patients with chronic lymphocytic leukemia *Leukemia & Lymphoma* (2022) Jun;63(6):1387-1397.)

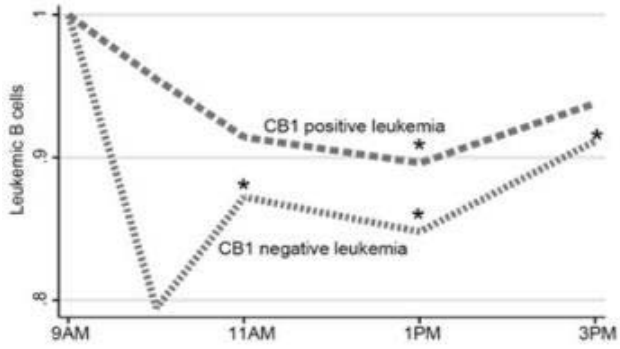

Figure 13 Reduction of leukemic B-cells after THC/CBD in CB1 positive (dashed gray line; n = 17) and CB1 negative (dotted gray line; n = 6) cases. One asterisk (*) indicate significant changes at the day with treatment, with respect to baseline with $p < 0.05$. (reprinted with permission from C. M. Melén *et al.* Clinical effects of a single dose of cannabinoids to patients with chronic lymphocytic leukemia *Leukemia & Lymphoma* (2022) Jun;63(6):1387-1397.)

Paper II: Δ9-THC and CBD could be detected in plasma, OF, and exhaled breath in all 23 patients. Δ9-THC and CBD could be detected for a longer time in OF and exhaled breath than in blood (Figure 14). Urine analysis detected the Δ9-THC metabolite (THC-COOH) 7 days after administration, also in a patient who received the low dose of 8.1/7.5 mg Δ9-THC/CBD. Relative ease of sample collection in combination with high sensitivity makes OF and exhaled breath a valuable addition when samples are handled correctly by trained personnel .

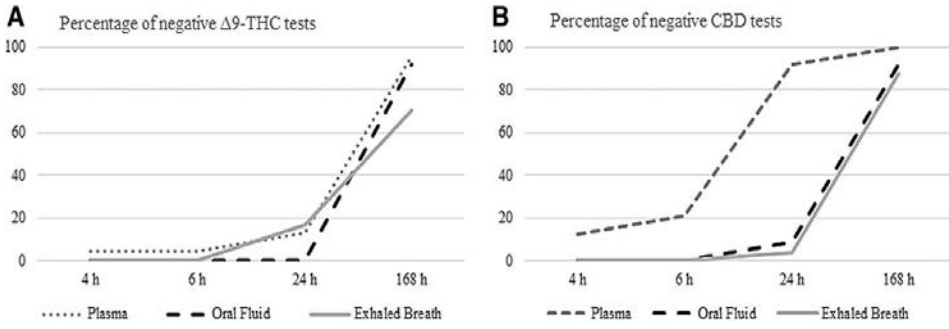

Figure 14 Visualization of increase of negative tests for (A) Δ9-THC and (B) CBD. Time points 1 and 2 h are omitted since the frequency of negative tests was 0% for all matrices at those time points, and thus, there was no difference to plot. (reprinted with permission from C. M. Melén et al. Δ9-THC and CBD in Plasma, Oral Fluid, Exhaled Breath, and Urine from 23 Patients Administered Sativex *Cannabis Cannabinoid Res* (2023) Apr 19.)

Paper III: 2-AG induces chemotaxis in 20 out of 22 primary CLL cell samples and 3 out of 5 primary MCL cell samples (Figure 15). 2-AG also induced, in a dose-dependent manner, migration of JeKo-1 cell line via CB1 and CB2 (Figure 16). 2-AG affected the CXCL12-mediated chemotaxis but did not impact the expression or internalization of CXCR4. We also show that 2-AG modulated p38 and p44/42 MAPK activation (Figure 17). These results suggest that 2-AG has a previously unrecognized role in mobilization of lymphoma cells by effecting the CXCL12-induced migration and the CXCR4 signalling pathways, and that the effects differ in MCL compared to CLL.

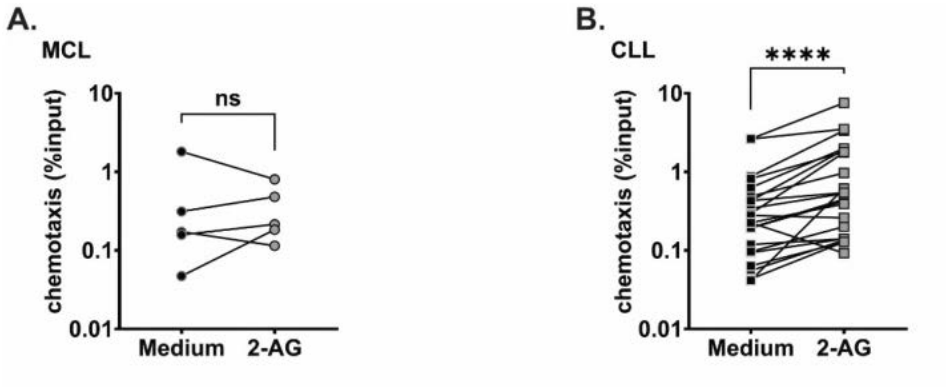

Figure 15 Chemotaxis towards 2-AG and cannabinoid receptor expression in MCL and CLL. (**A**) MCL (n = 5) and (**B**) CLL (n = 22) cells were subjected to chemotaxis towards vehicle or 2-AG, number of migrated cells is shown as percentage of input (log10 scale) at 4 h time point, line represents paired samples, ns: non-significant, **** $p < 0.0001$. (reprinted with permission from M. Merrien et al. 2-arachidonoylglycerol Modulates CXCL12-Mediated Chemotaxis in Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia *Cancers* (2023) 2023 Mar 3;15(5):1585.)

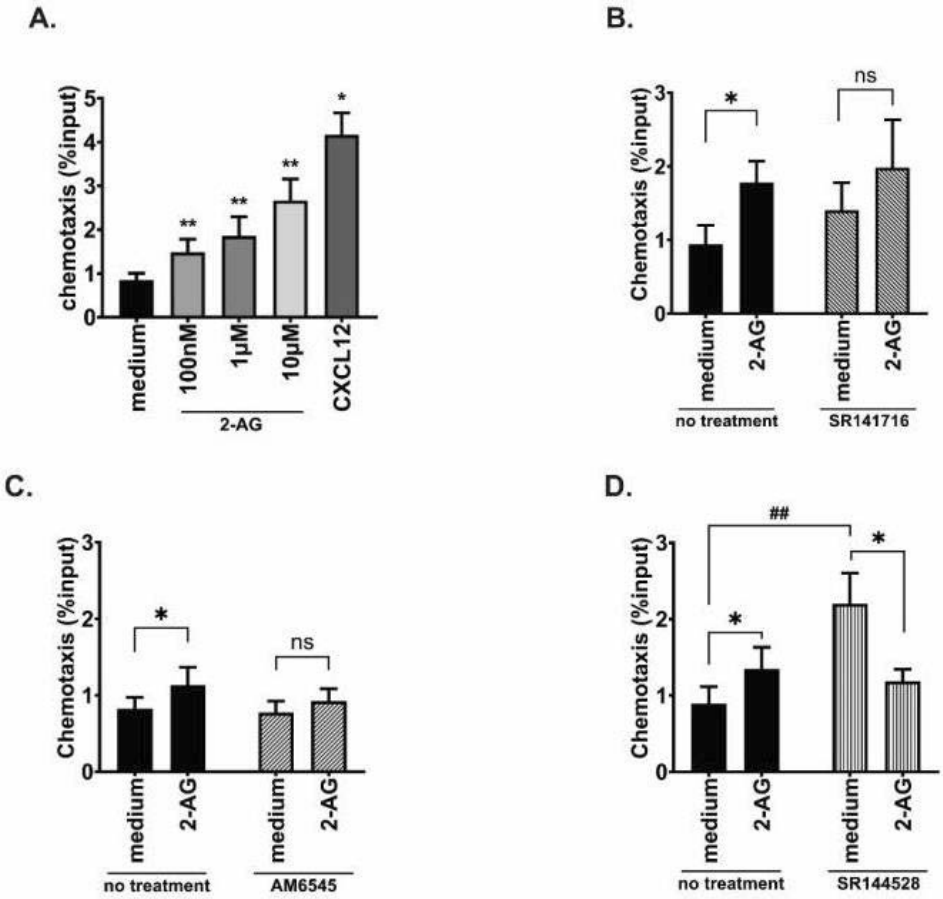

Figure 16 2-AG-mediated chemotaxis in MCL cell line JeKo-1. (**A**) JeKo-1 cells were subjected to chemotaxis towards vehicle or 2-AG at three different concentrations: 100 nM, 1µM and 10µM, or CXCL12 (200 ng/mL) as a positive control of chemotaxis, bars represent average of seven experiments and error bars are standard deviation; Paired *t*-test, * $p < 0.05$, ** $p < 0.01$. (**B**–**D**) Chemotaxis assays towards vehicle or 2-AG (100 nM) after JeKo-1 cells were incubated for 20 min with the CB1 antagonists (**B**) SR141716 (10 nM; n = 4), (**C**) AM6545 (10 nM; n = 5), or (**D**) with the CB2 inverse agonist SR144528 (10 nM; n = 8); Paired *t*-test, * $p < 0.05$, ns: non-significant, Wilcoxon matched-pairs signed rank test, ## $p < 0.01$. (reprinted with permission from M. Merrien et al. 2-arachidonoylglycerol Modulates CXCL12-Mediated Chemotaxis in Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia *Cancers* (2023) 2023 Mar 3;15(5):1585.)

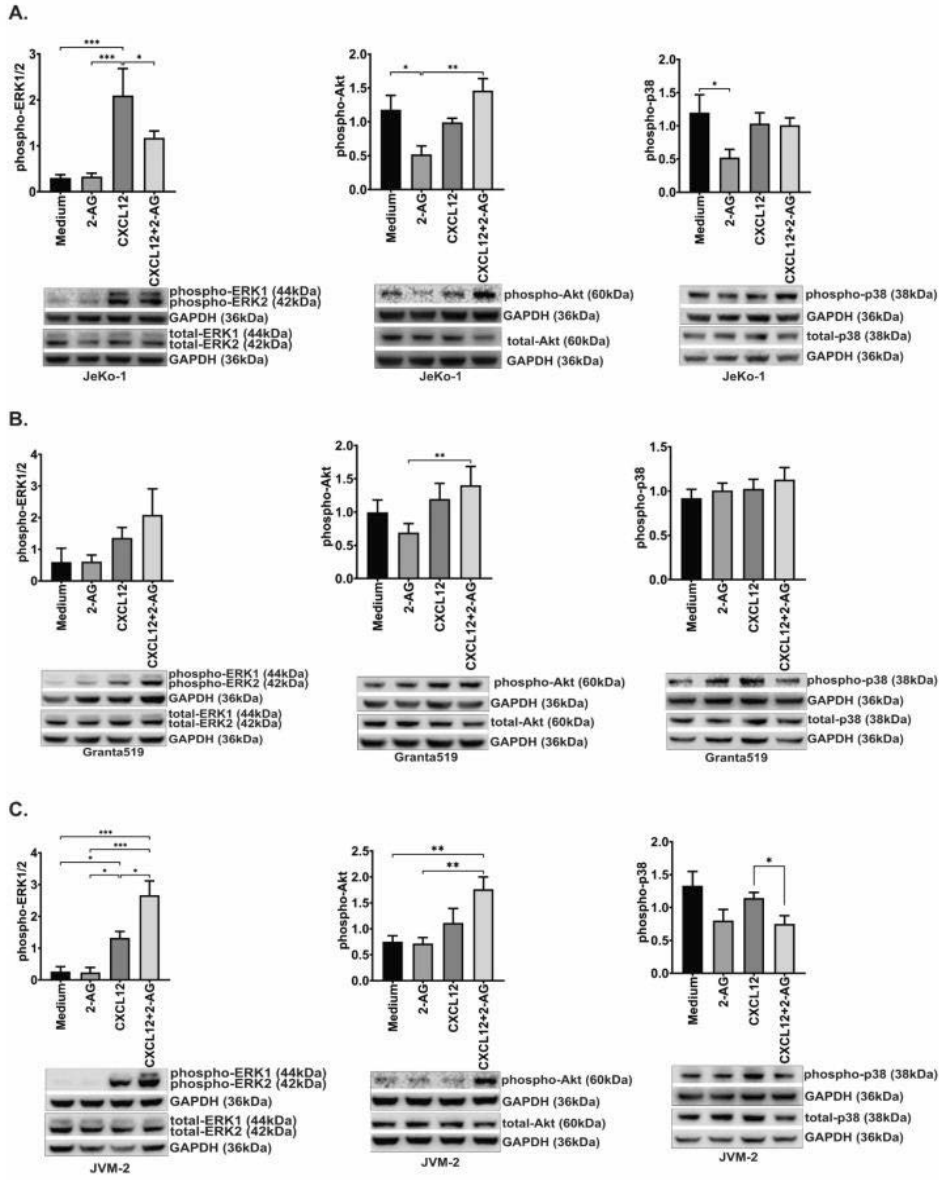

Figure 17 Activation of signalling pathways. (**A**) JeKo-1, (**B**) Granta519 and (**C**) JVM-2 were incubated for 2 min with CXCL12 (200 ng/mL) or 2-AG (100 nM) or the combination, and the activation of the signalling pathways ERK1/2, Akt and p38 was assessed by Western blotting, normalizing phospho/GAPDH band intensity to total/GAPDH, bars represent an average of the ratio of at least four repeats and error bars are standard error of the mean; paired *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant, a representative blot for each experiment is shown. (reprinted with permission from M. Merrien et al. 2-arachidonoylglycerol Modulates CXCL12-Mediated Chemotaxis in Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia *Cancers* (2023) 2023 Mar 3;15(5):1585.)

Paper IV: We compared the mRNA expression levels of sampling at the two-, four- or six-hour-timepoint with the zero-hour timepoints and found no significant changes and likewise we saw no significant changes between timepoint zero on control day and timepoint zero on intervention day. We did see significant changes of gene expression during the control day at the four-hour timepoint (Figure 18). We could also see significant changes in gene expression during the control day if we identified the various timepoints of maximum reduction in those patients that achieved a predefined reduction of lymphocytes of 15% (Figure 19). After identifying the overexpressed genes, we could see that several genes are connected to the AP-1 complex and PI3K-pathway which are previously known to be able to regulate lymphocyte activity and cell migration. When comparing the timepoints during the day patients received study drug, we could not see any significant changes in gene expression, indicating that the faster reduction that day was not mediated by transcription-changes. Instead, we can speculate on how the complex cross-talk and down-stream signalling between cannabinoid receptors and chemokine receptors influence the behaviour of the malignant B-cells.

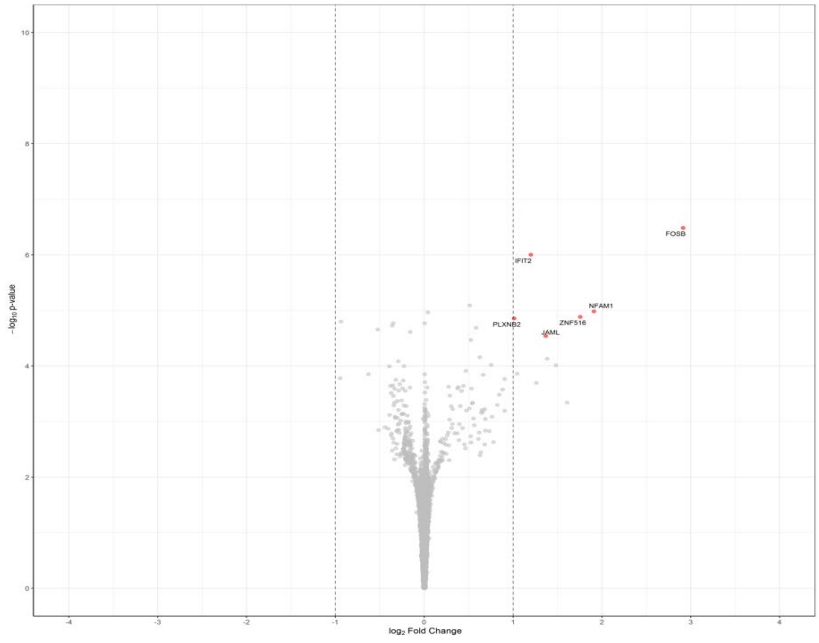

Figure 18 Volcano plot demonstrating changes in gene expression over the control day – comparing 4 hour control day versus 0 hour. Genes overexpressed with adjusted p ≤ 0.05 in combination with a log2-fold change of mRNA outside of -1 to 1 are shown with names written out.

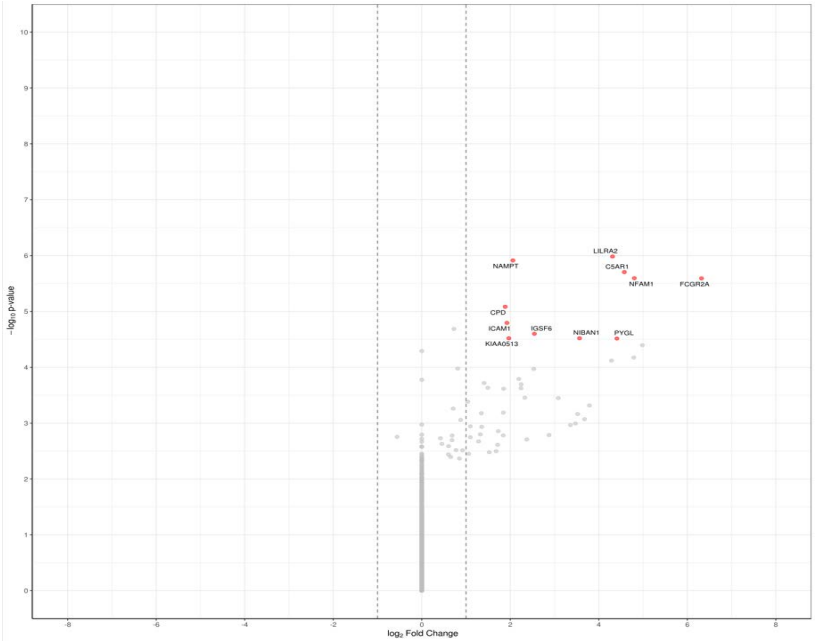

Figure 19 Volcano plot demonstrating changes in gene expression in patients with a profound decrease of lymphocytes on control day when comparing the timepoint of maximum decrease and timepoint zero hour. Genes overexpressed with adjusted $p \leq 0.05$ in combination with a log2-fold change of mRNA outside of -1 to 1 are shown with names written out.

5 DISCUSSION

In this thesis I have explored what effects cannabinoids have on indolent B-cell lymphomas. Based on pre-clinical experiments we designed a clinical study with a pragmatic approach that allowed us to build a cohort of patients with an easy-to-access disease since we included only patients with a leukemic spread of their lymphoma. The study drug we chose to use was an already approved compound of whole-plant cannabis with a fixed ratio of Δ9-THC and CBD. The rational for choosing this drug to investigate was foremost one of availability – using an already approved product outside of its formal indication is generally easier than to apply to use untested products in humans. This did however limit us in what we could investigate since we could not define which cannabinoid had which effect and at what concentration. If possible, it would have been of high interest to construct a multi-arm study with patients receiving only Δ9-THC in one arm, only CBD in a second arm and then a mixture of Δ9-THC/CBD in a third arm. This would have allowed us to possibly discern the separate effects of the cannabinoids and also to investigate their interaction more in detail.

Our method of reaching maximum tolerated dose meant that we could fairly quickly reach the doses of Δ9-TCH and CBD where we could hope to see relevant effects based on previous studies and still ascertain a high safety for our patients. What we could not achieve with this approach was a proper analysis of any dose-response since the cohorts receiving one, three and give actuations were too small.

It would also have been of great interest to also have sampled the lymph nodes of the study patients (in the cases where such disease was present). Simultaneous testing of peripheral blood and lymph nodes could have provided us with evidence of a relocation of cannabis-affected malignant cells from the blood stream to lymphatic organs. If such a population could have been identified then RNA-seq of that population could then possibly have revealed why and how that subgroup responded with relocation to lymph nodes after exposure to the cannabinoids.

In paper III, we try to systematically discern what chemotactic effects can be seen after stimulation of CB1 and CB2, together and individually, by the endocannabinoid 2-AG. A limiting factor was the relatively low prevalence of leukemic MCL-patients, which made it difficult to source primary MCL-cells, as well as the lack of a functioning CLL-cell lines. Thus, we could see significant

effects in primary CLL-cells but where then forced to use MCL-cell lines to further investigate potential underlying mechanisms. During this study we tested several new antibodies for detecting CB1 and CB2 and we identified reliable monoclonal antibodies which seem to be specific for CB1 and CB2. This will make it possible to investigate the surface expression of the receptors in addition to measuring the expression of the genes coding for the receptors at the mRNA level.

6 CONCLUSIONS

Paper I: We conclude that there were no signs of apoptosis in the malignant indolent lymphoma cells after a single administration of cannabinoids. The dose administered induced considerable and frequent AEs. We surmised that the study drug induced a redistribution of malignant cells away from peripheral blood and, most likely, to lymphoid tissue such as lymph nodes or bone marrow. If so, using cannabinoids concomitant with lymphoma treatment might result in a poorer outcome and thus we advise against this.

Paper II: We conclude that detection of THC and CBD in OF and captured breath were both good options to the invasive golden standard of detection in plasma. OF and captured breath had their drawbacks that need to be managed in future clinical studies and when applied to workplace testing and law enforcement. THC-COOH, a metabolite to THC was detectable in urine for a longer time than previously described in cannabis-naïve users.

Paper III: We conclude that there is an interplay between CR1 and CR2 when stimulated by 2-AG and that this interplay could affected how malignant cells home to various tissues. The two chemokines 2-AG and CXCL12 had different effects on MCL and on CLL-cells which further highlights the complexity of cell migration and tissue homing since it is a system in which a multitude of factors are involved.

Paper IV: We conclude that the most affected cells during the intervention day are likely not properly analysed by the RNA-seq since they have egressed away from the peripheral blood. During the control day, genes involved in the AP-1 complex and PI3K-pathway are significantly overexpressed in patient with profound decrease which might indicate a role for them in tissue homing and possible diurnal rhythm of CLL-cells.

7 POINTS OF PERSPECTIVE

Even though we could answer our most important and fundamental question with the clinical study in paper I and determine that there was no antineoplastic effect of cannabinoids on the indolent lymphoma cells in this experimental setting, there are several more questions left unanswered. Due to the complexity of the endocannabinoid system, and it's not yet fully mapped out down-stream regulation, it is difficult to ascertain which effect is induced by what cannabinoid and through which receptor. Since frequent anecdotal data and case reports exists of patients with lymphomas using recreational *Cannabis s* and then experiencing remissions, it had its merits to use a whole-plant extract of *Cannabis s*. in our clinical study as a pragmatic way to examine its effects. But the lack of single-cannabinoid drugs left us without the possibility to properly investigate what effects are induced by what cannabinoid *in vivo*. Instead, we did more *in vitro* experiments in paper III, using both primary cells and MCL cell lines and the endocannabinoid 2-AG to try and discern how stimulation of either CB1 and/or CB2 affected the cells capability to undergo chemotaxis. It would have been interesting to not only test 2-AG but also the study drug used in paper I and II. That could have provided us with an *ex vivo*-model for the supposed migration of lymphocytes that we likely saw in paper I. The main focus of paper III was to try and discern the various mechanisms by which an agonist to CB1 and CB2 induced migration and since the study drug from paper I was whole plant *Cannabis s.,* with over 120 potential ligands for the cannabinoid receptors, it was considered not appropriate to include it in the study. Also, when paper III was planned, we didn't know the results of paper I and that whole plant *Cannabis s* . likely induce migration. In retrospect, it would have been of interest to pragmatically investigate Sativex in a chemotaxis experiment. When the later part of the experiments for paper III was being performed, we had identified antibodies to detect CB1 and CB2 protein but had not yet developed a suitable method for performing additional experiments using for instance proximity ligation assays to fully investigate the possibility of CB1-CB2 heterodimerisation. Investigating this and additional formation of inert or functional GPCR-heterodimers could be an important step to further understand how the ECS functions and how it interplays with the various other GPCRs. Since there are few cells, that we know of today, that express both CB1 and CB2 in significant amounts on its cell surface, the possible formation of CB1-CB2 heterodimers on

MCL and CLL cells could even be a favourable target if a dual-anchoring antibody could be constructed.

Paper II stands out as a work of its own with very practical implications for handling non-invasive drug detection kits which are becoming more frequently used in for example workplace testing and also in the hands of traffic police. The method to capture breath could also be of interest for a wider range of use since it is actually not breath but fragments of alveolar cells that are being analysed. These fragments could very well also be used to detect proteins specific for infectious pathogens such as fungi, viruses and bacteria and could then be used as an analysis for deep respiratory infections when diagnostic bronchoscopy is not advisable.

For paper IV, we discussed first to use phosphoproteomics to analyse our collected cells. This method is appealing since it can more accurately identify rapid intracellular changes such as activation of signalling pathways. This could have resulted in finding effects of the administered study drug on the circulating lymphocytes before they, supposedly, egressed away from the blood stream. The drawback of the method is that it requires more viability frozen cells than the RNA-seq we eventually choose to use and thus not all patients in the study would have been eligible for analysis. Furthermore, the method was not yet fully set up at the proteomics core facility. We will now move on with mixed effects modal-testing of the data to further try and find trends and changes that wasn't detected in the first step of the RNA-seq analysis.

8 ACKNOWLEDGEMENTS

I would like to thank all those that have participated in the completion of this thesis. In particular, I would like to thank:

Björn Wahlin who casually wondered if I thought it "might be fun to look at what cannabis does to lymphomas" when I brought up some questions about possible research projects. As with many things, you were right.

Birgitta Sander for allowing me to be a part of her group. You always made me feel welcome even when the clinical reality or parental leaves had kept me away from PhD-life for long periods of time. Your utter professionalism combined with humility is inspiring.

Agata Wasik and **Magali Merrien,** I owe you thanks for your patience with my untrained hands in the lab and for always offering your time, help and great explanations whenever problems arose. This work absolutely would not have been possible without you.

Kristina Sonnevi for being a great colleague and my on-and-off boss during residency and again in the lymphoma department. Working magic with the schedules to fit in my courses and adjusting on the fly to make room for me to combine clinical rotations with my projects. And for keeping a much-appreciated watchful eye over Björns doings and things that should be done but was not.

Martin Jädersten for being my clinical supervisor during residency. You inspired me to always be curios and your dedication is amazing.

Henna-Riikka Junlén, Birger Christensson, Olof Beck and Georgios Panagiotidis for great cooperation with the articles.

All my great colleagues at the Hematology department! I enjoy every day with you all, I can hardly imagine a better group of people to do this job with. I look forward to seeing you all in the sauna.

Torsten Dahlén for stepping up as the head of lymphoma section and doing a great job at it!

Marit Hoglund for giving me my introduction-papers and setting me up in 2013 when I started at the Hematology Department in Huddinge. And for still being here, running basically everything behind the scenes.

All the **nurses** and the **care staff** in our wards and out-patient clinics, together we try our best to help and often we succeed. A special thanks to **Eva Eriksson**, **Elisabeth Allenbrant**, **Sandra Lindé** and **Sofia Dovborg** in Solna for your outstanding work with the patients in my care. A special thanks also to **Hannele Kleemola** and **Katarina Malmsten** in Huddinge for your support and friendly words during my years there.

All the **patients** who participated in the studies. You did not have to do it; I hope you do not regret it and I want to believe that this work will be of benefit to the patients that came after you.

All my **friends,** old and new. I am so happy to have you. For adventures had and for those to come.

Glamorama for providing me the opportunity to meet and share experiences with a most unlikely gathering of great people.

My **mother**, **sister, Ragge** and all my **aunts** and their great families. It is amazing to have so many people in my life that supports me and my family so much. Looking forward to enjoying the world cup (in Madeira?) again.

Louise for proof-reading my papers and listening to my presentations. Again. And again. And again… For always offering help and support. For understanding and caring.

Liv and **Karin** for being the wonderful persons that you are. For teaching me so much about the world and myself. I am so happy to have you and look forward to seeing you grow up to become whoever you will be.

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