Peripheral blood, Liver and Intestine-specific Lymphocytes in Primary Sclerosing Cholangitis and Inflammatory Bowel Disease



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Thesis submitted for the degree of Doctor of Philosophy in Clinical Medicine

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Abstract

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Primary sclerosing cholangitis (PSC) is an immune-mediated fibroinflammatory condition of the biliary system which often carries a poor prognosis. Despite significant research efforts, there is no known medical therapy which can slow the progression of disease. It is linked with inflammatory bowel disease (IBD) and there are some limited data which suggest a role for gut-homing lymphocytes in the pathophysiology of the disease.

I revealed via flow cytometry that patients with PSC had a reduced proportion of CCR9+ T-cells and mucosa associated innate-like T-cells (MAITs) within the peripheral blood as compared with controls. Furthermore, I was able to demonstrate an immunological difference within the ileum of patients with PSC/IBD as compared with IBD alone; namely that there is a reduced proportion of CCR9+ T-cells in the PSC ileum, lending evidence to the notion that the IBD in PSC is its own immunologically distinct disease.

Utilising the technique of fine needle aspiration (FNA) of the liver, I found there was no difference in the proportion of gut-homing T-cells (i.e. CCR9+ or β7+) within the livers of patients with PSC compared with liver controls (LC). However, a subset of patients in both PSC and LC cohorts had a relative abundance of hepatic CCR9+ T-cells, though no correlation could be found with fibrosis or inflammatory measures. Patients with PSC, however, had higher hepatic proportions of MAITs and CD69+ CD8+ memory T-cells compared with LC, indicating a potential role in the pathophysiology of the disease. I was able to demonstrate that FNA liver could successfully be used for single cell RNAseq.

Vedolizumab (VDZ) is a monoclonal antibody which targets α4β7 on T-cells, thereby theoretically preventing migration of lymphocytes to the gut. It is a licensed therapy in IBD but its use in PSC has not been established. I was able to demonstrate for the first time in humans that VDZ does indeed reduce the proportion of β7+ T-cells within the colon, establishing proof of concept. Furthermore, CCR9+/β7+ CD4+ T-cells rise within the peripheral blood with VDZ indicating their being prevented from leaving the circulation.

There was a heterogenous clinical hepatic response to VDZ among 102 patients with PSC/IBD, as evaluated by change in alkaline phosphatase (ALP). Around 20% of patients with PSC/IBD has a clinically significant drop in ALP of >20% from baseline. However, overall, the ALP rose with VDZ by a small amount. The IBD in PSC responded to a similar beneficial degree as in IBD alone, supporting its use for active IBD in PSC.

A subset of patients with PSC had a significant rise in their ALP with VDZ and were investigated with liver biopsy, which revealed an increased proportion of T-cells which were CCR9+ compared with patients not on VDZ. These data suggest that in some patients with PSC, VDZ may cause a redistribution of gut-homing T-cells from the gut and peripheral blood to the liver, which has not been apparent among patients with IBD alone, though further investigation is warranted.

Overall, this thesis provides a comprehensive analysis of peripheral blood, intestinal and hepatic lymphocytes in PSC compared with controls, as well as evaluating the effects of VDZ in these settings at a clinical and cellular level.

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Abbreviations

5-ASA	5-aminosalicylic acid
AIH	Autoimmune hepatitis
ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APC	Antigen presenting cell
ATRA	All-*trans*-retinoic acid
BSA	Bovine Serum Albumin
CCL25	chemokine (C-C motif) ligand 25
CCR9	CC chemokine receptor 9
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
CRC	Colorectal cancer
D-PBS	Dulbecco's phosphate buffered saline
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DSS	Dextran sodium sulphate
EDTA	Ethylenediaminetetraacetic acid
ERCP	Endoscopic retrograde cholangiopancreatography
FACS	Flow assisted cell sorting
FcR	Fc Receptor
FCS	Foetal calf serum
FFPE	Formalin-fixed, paraffin-embedded
FMO	Fluorescence minus one
FNA	Fine needle aspiration
FSC-A/H	Forward Scatter-Area/Height
GALT	Gut associated lymphoid tissue
GEM	Gel Bead-In-Emulsion
GETAID	*Groupe d'Etude Thérapeutique des Affections Inflammatoires du tube Digestif*
GGT	γ-glutymyltranspeptidase
GWAS	Genome-wide Association Studies
HC	healthy controls
HEPES	4-(2-hyroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPF	High power field
IBD	Inflammatory bowel disease
IBD-u	IBD-unspecified
IFN-γ	Interferon-γ
IFX	Infliximab
IHC	Immunohistochemistry
IL	Interleukin
IPAA	Ileal pouch anal anastomosis
IPSCSG	International PSC Study Group
ITGA4	Integrin α4
ITGB7	Integrin β7
IU/L	International units/litre

Abbreviations …*continued*

LAG-3	Lymphocyte activating gene-3
LC	liver controls
LIL	Liver infiltrating lymphocytes
LPMC	Lamina propria mononuclear cells
LSEC	Liver sinusoidal endothelial cell
LSM	Liver stiffness measurement
LT	liver transplantation
MACS	Magnetic assisted cell sorting
MAdCAM-1	Mucosal Addressin Cellular Adhesion Molecule-1
MAIT	Mucosal-associated invariant T-cell
MES	Mayo Endoscopic Subscore
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MR1	MHC-like protein 1
MRCP	Magnetic resonance cholangiopancreatography
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NI	Nancy Index
NK	Natural killer
NKG7	Natural Killer Granule Protein 7
PBC	Primary biliary cholangitis
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PSC	Polymerase chain reaction
PSC	Primary sclerosing cholangitis
PT	Prothrombin time
RAG-1	Recombinase activation gene-1
REC Ref	Research Ethics Committee Reference
RPMI-1640	Roswell Park Memorial Institute-1640
scRNAseq	Single cell RNA sequencing
SD-PSC	Small-duct primary sclerosing cholangitis
SES-CD	Simple Endoscopic Score for Crohn's Disease
SSC-A	Side Scatter-Area
sVAP-1	Soluble vascular adhesion protein-1
t-SNE	t-Distributed Stochastic Neighbour Embedding
T_{CM}	Central memory T-cells
TCR	T-cell receptor
TECK	Thymus expressed chemokine
T_{EM}	T-effector memory cells
T_{EMRA}	Terminal effector memory T-cells
T_H	T-Helper
TI	Terminal ileum
TNF-α	Tumour necrosis factor-alpha
T_{reg}	T-regulatory
T_{RM}	Tissue resident memory T-cell
UC	Ulcerative colitis
UCEIS	Ulcerative Colitis Endoscopic Index Of Severity

Abbreviations …*continued*

UDCA	Ursodeoxycholic acid
ULN	Upper limit of normal
UMAP	Uniform Manifold Approximation and Projection
VAP-1	Vascular adhesion protein-1
VCAM-1	Vascular cell adhesion molecule-1
VDZ	Vedolizumab
WCHG	Wellcome Centre for Human Genetics

List of Publications derived from this work:

1. **Lynch KD**, Chapman RW, Keshav S, et al. *International Experience of Vedolizumab in Patients with Primary Sclerosing Cholangitis and Inflammatory Bowel Disease*. Clinical Gastroenterology and Hepatology 2019. *Manuscript submitted and under review.*
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1 Introduction

1.1 Overview

Gut-homing lymphocytes are integral to the surveillance and protection of human small and large intestines. They are a specific subset of T-cells which preferentially migrate from the peripheral circulation to the bowel through an intricate system of tethering, rolling, firm adhesion and diapedesis into the intestinal lamina propria, known as the "adhesion cascade".[1, 2] This pathway is dependent on various chemokines and adhesion molecules present on intestinal endothelium and chemokine receptors and integrins expressed on T-cells and various other immune cells. Dysregulation of intestinal immune surveillance can lead to inflammation and tissue destruction, such as occurs in inflammatory bowel disease (IBD). Disruption of the adhesion cascade in this circumstance can lead to therapeutic benefit in this setting.

Meanwhile, aberrant expression of gut-homing molecules has been described within the liver in the setting of the rare liver disease, primary sclerosing cholangitis (PSC). Lymphocytes supposedly destined for the bowel have been identified within the liver in PSC, a condition which itself is associated with intestinal inflammation. However, given the rarity of PSC, there is limited knowledge of the extent of the involvement of these gut-homing lymphocytes in PSC/IBD, within the peripheral blood, bowel and liver.

The focus of this thesis is to explore the presence of these gut-homing lymphocytes in the various tissue compartments in PSC and IBD as well as to identify the effect of manipulating this pathway with therapeutic agents targeting this pathway.

1.2 Primary Sclerosing Cholangitis

1.2.1 Epidemiology of PSC

Primary sclerosing cholangitis (PSC) is a progressive inflammatory condition of the liver leading to stricturing and dilatation of the biliary tree. It is closely associated with inflammatory bowel disease (IBD), with approximately 70% of patients with PSC having concomitant IBD.[3] It is a rare disease, affecting up to 16 per 100,000 population, with the incidence thought to be increasing over time.[4] It carries a poor prognosis with mortality rates quoted as high as 28% at 6 years, and currently there are no effective therapies which can slow its progression.[5] Liver transplantation remains the mainstay of treatment, however, although 5-year survival rates after transplantation for PSC are greater than 80%, it is not without significant morbidity and mortality, and resources for transplant are limited.[6] Furthermore, around a quarter of patients have a recurrence of PSC in the transplanted organ, hence the need to find targeted and beneficial medical therapies.[7, 8]

1.2.2 Clinical features and diagnosis of PSC

The majority of patients with PSC are asymptomatic at diagnosis, and are brought to attention due an incidental finding of elevated liver biochemistry on a routine blood test in a patient with inflammatory bowel disease.[9] It affects males more than females at approximately 2:1, and can present as early as childhood through to older age, with the median age at diagnosis around 40 years.

To make a diagnosis, a patient should have evidence of cholestasis, such as an elevated alkaline phosphatase (ALP) together with an elevated γ-glutymyltranspeptidase (GGT)

with a cholangiogram exhibiting characteristic features of strictures and/or beading within the intra- and/or extrahepatic biliary system. This is usually best demonstrated by magnetic resonance cholangiopancreatography (MRCP) but occasionally is shown on endoscopic retrograde cholangiopancreatography (ERCP). In the setting of a normal MRCP in a patient with IBD and cholestatic liver biochemistry, a liver biopsy may be performed.

Characteristic features on liver biopsy in PSC include lymphocytic portal inflammation, concentric fibrosis surrounding the bile duct (so called “onion skin” fibrosis) and on occasion, bile duct destruction (“ductopenia”). PSC is a patchy disease, so liver biopsy may be falsely negative. If a patient has a normal cholangiogram but characteristic features of PSC on liver biopsy, they are said to have “small duct-PSC” (SD-PSC), a condition which carries a much more favourable prognosis. SD-PSC may progress to classical PSC (as seen by the development of an abnormal cholangiogram) in 20-25% cases, which then confers the same prognosis as classical PSC.

Symptoms include fatigue, right upper quadrant abdominal pain, pruritis, and attacks of biliary cholangitis. The latter is an infection in the bile duct brought about by strictures leading to biliary stasis and subsequent infection, causing abdominal pain, fevers and general malaise. It can be treated with antibiotics, which may need to be intermittent or long-term.

In latter stages of disease, patients may become jaundiced due to their biliary strictures, at times necessitating the need for endoscopic biliary stenting, and as the disease

progresses, patients develop various signs of liver failure, including complications of portal hypertension such as ascites and variceal bleeding, coagulopathy and encephalopathy.

1.2.3 Pathophysiology of PSC

The primary injury seen on liver biopsy in PSC is a mixed inflammatory infiltrate of lymphocytes, plasma cells and neutrophils, initially confined to the portal tracts in early disease.[10] T-cells are the predominant cell involved in the infiltrate, making up to 80% of the mononuclear cells, and slightly more CD4+ T-cells than CD8+ T-cells, which is the opposite of what occurs in the healthy liver.[11, 12] The focus of inflammation is most intense surrounding the bile ducts, and often there is an interface hepatitis, where there is an inflammatory infiltrate associated with hepatocyte necrosis at the junction between the hepatic lobules and the portal tracts. Persistent inflammation leads to bile duct destruction and peribiliary fibrosis, which progresses to disorganised ductular proliferation and bile duct loss. Eventually, fibrosis and inflammation extend through the liver and lead to bridging fibrosis and cirrhosis. Various histological scoring systems have been evaluated in PSC, and the Nakanuma staging system has been independently associated with clinical outcome.[13]

Genetic predisposition is evident in that first-degree relatives of patients with PSC have an 11-fold increased risk of developing PSC.[14] Furthermore, as identified in North American and European Genome-wide Association Studies (GWAS) and immunochip studies, there are several genetic susceptibility loci associated with PSC.[15-21] The strongest association is with the genes associated with recognition of antigens by the immune system – the human leukocyte antigen (HLA) complex – and therefore implies a

role for the adaptive immune system in development of PSC. HLA genes represent those which make up the major histocompatibility complex (MHC) which are present on cells within the human body (discussed further below in 1.4). The main attributable genetic risk for PSC is at *HLA-B*, which is a class I HLA, though it is with a complex association signal that implies the HLA class II region, specifically, *HLA-DRB1*.[22]

The MHC Class I (present on all human cells) and Class II molecules (normally present only on antigen presenting cells) are responsible for presenting intracellular and extracellular antigenic peptides respectively to the T-cell receptor (TCR) on CD4+ and CD8+ T-cells. The antigen presented may be pathogenic or self, and the interaction between the MHC and TCR results in either a proinflammatory or tolerant reaction respectively. In the setting of autoimmune disease, such as PSC, it is hypothesised that loss of self-tolerance may lead to unbridled immune reactivity. The strong association of PSC with particular HLA haplotypes indicates that PSC may be an antigen-driven disease in genetically susceptible individuals. However, as in other HLA associated diseases, despite knowing the specific susceptible HLA, the actual antigenic peptides remain unknown, and antigen-finding studies, whilst ongoing, have proven so far to be extremely difficult.

Overall, 22 susceptibility loci have been identified as associations with the development of PSC, in addition to the HLA associations.[21] The majority of these encompass genes mainly involved in adaptive immunity rather than innate immunity, which together with the predominance of CD4+ T-cells within the liver biopsies of patients with PSC, signify an important role for CD4+ T-cells in the pathogenesis of PSC.

Interestingly, very few IBD-associated genes overlap with PSC-associated genes, and only around half of PSC genes are associated with IBD.[23] This suggests that the IBD in PSC may represent a unique subtype of IBD, which goes along with distinct clinical features in PSC-IBD which will be further discussed below.

Despite these key advances in the understanding of the genetic associations with PSC, there has been little advance in the understanding of the implicated altered immunological consequences in PSC. This is in part due to the rarity of PSC, and furthermore, the difficulty in obtaining liver-derived samples in PSC. This challenge will be addressed in this thesis with a novel technique for accessing liver immune cells – fine needle aspiration of the liver.

Another component of the theory surrounding the pathogenesis of PSC is that of the "leaky gut theory". Under normal homeostatic conditions, gut commensals and pathogens are restricted to the gut by mesenteric lymph nodes.[24, 25] It is possible that with increased gut permeability as evidenced in IBD, bacterial products can be exposed via the portal circulation to the liver sinusoids, leading to Kuppfer cell and liver sinusoidal endothelial cell (LSEC) activation by bacterial toll like receptor ligands. Potentially this could lead to a molecular mimicry autoimmune-like reaction in the setting of altered adaptive immune predisposition.

Finally, there are implications for gut-homing lymphocytes trafficking to the liver as the cause of PSC, which will be discussed further below.

1.2.4 Prognosis of PSC

As mentioned earlier, there is significant morbidity and mortality in PSC. The median survival reported varies in the literature. Initially thought to be 10-12 years, it is more likely to be around 21 years, as demonstrated in a large Dutch cohort which included patients from non-transplant centres as well.[26] Malignancy is common in PSC, occurring in up to 25% of patients.[5] Malignancies associated with PSC include colorectal cancer, cholangiocarcinoma, hepatocellular carcinoma and gallbladder carcinoma.

The risk of progressing to requiring a liver transplantation and/or death in PSC can be determined by various risk-score calculators.[27-29] The majority include evaluation of the level of ALP within the blood over time, as well as the bilirubin level. There are a number of recent studies which have shown that a normal ALP, or reduction to lower levels such as below 1.5 times the upper limit of normal (ULN), is associated with a good prognosis.[30] Likewise, persistently raised ALP has been associated with progression to death and/or liver transplantation. ALP is an incomplete surrogate marker, and there is controversy over the ideal surrogate endpoint in clinical trials of PSC, though the inclusion of ALP as an endpoint is almost universal.

1.2.5 Medical therapy in PSC

Ursodeoxycholic acid (UDCA), a hydrophilic bile acid which makes up 3% of endogenous bile acids, but may be supplemented with a synthetic form, is the most studied medical therapy in PSC. It has been shown to reduce liver biochemistry in 10 of 11 clinical trials, but no study has ever shown a survival benefit, nor beneficial improvement.[31] Furthermore, a study of high dose UDCA was associated with an increased rate of serious

adverse events.[32] Hence, practice varies around the world with regards to the use of UDCA in PSC.

Given the potential immune-mediated pathogenesis, multiple pilot studies and case series have been published of various immunosuppressants in PSC, all with negative results. These include azathioprine,[33] cyclosporine,[34] methotrexate,[35, 36] tacrolimus,[37] penicillamine,[38] colchicine,[39] and infliximab.[40]. One study has suggested a possible beneficial role of adalimumab, an anti-TNF agent, however this was a retrospective case series and only included 19 patients on the drug.[41]

Trials are currently in progress of various therapies targeting fibrosis, bile salt manipulations, and altering the intestinal microbiome, with results awaited. Furthermore, there is a study aimed at manipulating the gut-liver axis (discussed below).

1.3 Inflammatory bowel disease

1.3.1 Epidemiology of IBD

IBD is an idiopathic autoimmune disease which affects various portions of the gastrointestinal tract leading to abdominal pain, altered bowel function and gastrointestinal bleeding.[42] It is frequently disabling to patients, causing impaired quality of life, reduced work capacity and social isolation and it carries a significant cost and health burden.[43, 44] The prevalence is around 0.3-0.5% in Europe, and its incidence appears to be increasing with time.[45] It affects males and females equally, and onset can occur at any age, though the peak onset is between 15 and 35 years.[46, 47]

There are two specific major subtypes – Crohn's Disease (CD) and Ulcerative Colitis (UC), and though there are key distinguishing features between the two, they are thought to be part of a continuous spectrum. A minority of patients fall into a further third intermediate subtype termed "IBD-unspecified" where features of both CD and UC are present, and a definite diagnosis of one or the other cannot be made. Whilst there are multiple beneficial therapies licensed for IBD, patients usually require lifelong medication, frequently require surgery and there is no cure.

1.3.2 Clinical features in IBD

The predominant symptoms of CD are abdominal pain and diarrhoea, and a subset of patients with the more severe penetrating phenotype have draining fistulae and abscesses, particularly in the perianal region. In CD, inflammation can affect any part of the gastrointestinal tract from mouth to anus, though the ileum is almost always involved, whilst in UC, disease is restricted to the large bowel. Patients with UC more often experience bloody diarrhoea and urgency. In both diseases, if left untreated, progressive inflammation can lead to gastrointestinal perforation, a complication associated with significant morbidity and occasionally mortality, and in CD, bowel obstruction can also ensue, necessitating surgical bowel resection or strictureplasty.

Symptom scores exist to quantify the severity of patient's symptoms. However, there is a discordance between symptoms reported and the presence of actual biological inflammation.[48] This has led to an international effort for physicians to "Treat to Target", meaning that one uses objective measures to evaluate disease and assess treatment response in addition to symptomatic measures.[49, 50] Endoscopic scores exist

to quantitatively assess severity of intestinal inflammation including the Mayo Endoscopic Subscore (MES)[51] and Ulcerative Colitis Endoscopic Index Of Severity (UCEIS)[52] for UC and the Simple Endoscopic Score for Crohn's Disease (SES-CD)[53] for CD. Numerous histopathological indices have also been developed, including the Nancy Index (NI) in UC, which has been shown to correlate with UCEIS.[54] The use of endoscopic and histological scores is increasingly being used both in the clinical setting and as primary endpoints in clinical trials in IBD so as to accurately measure a patient's inflammatory response to a therapeutic intervention.

1.3.3 Pathophysiology of IBD

The chronic inflammation seen in IBD is a consequence of a dysregulated immune response within the gastrointestinal system. An individual's immune response within the bowel is thought to be a result of a combination of individual genetic make-up, environmental triggers, and the gut microbiota. Associations in all of these areas have been found with IBD.[55]

Multiple GWAS and immunochip studies have led to the discovery of 245 susceptibility loci associated with IBD.[55-57] The majority are shared across both forms of IBD, with a smaller number specific to either CD or UC. Many loci encompass various genes encoding adaptive and immune pathways and have led to the development of targeted biological therapies for IBD. These targets include tumour necrosis factor-alpha (TNF-α) and Interleukin-23 (IL-23), against which there are now beneficial monoclonal antibodies licensed for use in IBD. More recently, a susceptibility locus in the region of integrin α4

(ITGA4) has been identified, suggesting a potential role for integrins such as $\alpha4\beta7$ and $\alpha4\beta1$ in the pathogenesis of IBD.[56]

Distinct alterations in the gut microbiome have also been identified in patients with IBD, particularly in CD, implicating a role in the pathogenesis. Patients with CD have an increased abundance of Bacteroides and Proteobacteria and a decrease in abundance of Firmicutes, and in both diseases there is decreased bacterial diversity.[58-60] It has also been shown that patients with IBD have an enhanced immune reactivity against microbial antigens. Whether IBD-associated dysbiosis is a primary or secondary phenomenon is unclear, but it is certainly thought to be inherently linked with the development of IBD and subsequent activity.[61]

Environmental factors have been found to be associated with IBD. These include low sunlight exposure and Vitamin D deficiency, smoking (increased CD risk, decreased UC risk), antibiotic exposure in early life, and acute gastroenteritis in adulthood.[62-66] Environmental factors are also implicated in the fact that there is a lack of complete concordance in monozygotic twins, and there is an increased occurrence of IBD in spouses.[67, 68]

Numerous alterations in the function and phenotype of various leukocytes and immune pathways have been described in the blood and mucosa of patients with IBD as compared with healthy controls, both in mice and in humans. These include alterations to macrophage and dendritic cell function within the innate immune system, and shifts towards a more pro-inflammatory phenotype within the adaptive immune system.

Traditionally, CD was thought to be a T-Helper 1 (T_H1) driven disease and UC a T_H2 driven disease, but in the last decade, the emergence of a T_H17 pathway has been implicated in both UC and CD.[69, 70] More recently, a new T-Helper phenotype has been described – T_H9 – which has been demonstrated to play a role in driving UC.[71, 72]

1.3.4 Medical therapy in IBD

The goal of therapy in IBD is to abrogate inflammation and thereby reduce symptoms whilst minimising the development of therapy-related complications. The mainstay of treatment over the past many decades has been to suppress inflammation by reducing the host immune response. This is conventionally done in the form of corticosteroids (prednisolone), thiopurines (azathioprine and mercaptopurine), and other various immunomodulators such as methotrexate.[73, 74] Whilst these drugs have been shown to be effective in induction and maintenance therapy of both CD and UC, they have a significant side effect profile including a host of glucocorticoid-related complications and opportunistic infections. Milder forms of UC have been shown to respond to 5-aminosalicylic acid (5-ASA), which has minimal side effects, but a large proportion of patients require immune suppression.

The use of targeted therapies in the form of biological agents and more recently small molecules against particular immune proteins has risen over the past 20 years.[75] Many therapies have been developed as a result of their success in various rheumatological conditions such as rheumatoid arthritis and psoriasis, which share common pathophysiological mechanisms. The most commonly used biologic agents are infliximab and adalimumab, which target TNF-α; vedolizumab, which targets integrin $\alpha4\beta7$; and ustekinumab, which targets the p40 subunit of IL-12 and IL-23. More recently, the small

molecule, tofacitinib, has been licensed for use in UC, which targets the JAK-STAT pathway (Janus Kinases-Signal Transducer and Activator of Transcription proteins).

All of these agents augment the dysregulated immune response in IBD leading to clinical benefit in a portion of cases. Primary biologic failure and secondary non-response are common, and the chance of responding to a second or third biologic agent is reduced compared with when one is biologic-naïve, hence the need for personalised medicine and choosing the right drug for the right patient.[76-78] Whilst there is an abundance of drug development occurring within the IBD academic field, unfortunately remission rates in recent clinical trials to date remain disappointing. They are in the order of 25-40% compared with the excellent results recently obtained by hepatology colleagues in the arena of Hepatitis C which approach 100%.[79, 80]

1.3.5 IBD phenotype in PSC

As mentioned, the majority of patients with PSC have IBD, and the IBD usually, but not always, predates the PSC diagnosis. The majority of PSC-IBD is ulcerative colitis (approximately 80%) with around 15% having CD, and 5% IBD-u. Conversely, around 5% of patients with IBD develop PSC, though this might be underrepresented, as a recent study suggests subclinical PSC might be as high as 8%.[81]

Patients with PSC-IBD usually have a pancolitis (involvement of the whole bowel), with a predominance for right-sided inflammation, and a large proportion of patients have relative rectal sparing, which is uncommon for patients with UC alone.[3] Up to 40% of patients have “backwash ileitis” which is mild inflammation in the ileum presumably due to diminished ileocaecal valve function in the setting of inflammation allowing for

retrograde flow of colonic effluent and subsequent inflammation, and yet this is rare in UC without PSC.

Disease activity, despite the extensive distribution, is usually mild in PSC-IBD, and often there is a discord between the degree of inflammation endoscopically, and the minimal symptoms the patient perceives. Furthermore, if patients have a proctocolectomy and ileal pouch anal anastomosis (IPAA), patients with PSC-IBD have a higher rate of pouchitis and pouch dysfunction compared with those with UC alone.[82]

To date there are only two studies looking at the intestinal immunology in PSC-IBD as compared with IBD alone. A Swedish study examining colonic T-cells from patients with active PSC-UC (n=4) showed higher proportion of CXCR3+ CD8+ T-cells compared with quiescent PSC-UC (n=7), active or quiescent UC (n=5, 9 respectively) or healthy controls.[83] There was no difference in expression of CXCR3 on colonic CD4+ T-cells. They suggested that CXCR3 on CD8+ T-cells may serve as a chemotactic mechanism for homing to the liver; however, one might expect there to therefore be lower amounts of CXCR3+ T-cells in the PSC-IBD colon if this were the case, rather than higher, and also one might interpret these results with caution due to the small sample size.

A second study from a group at our institution demonstrated an increase in interferon-γ (IFN-γ) secreting T-cells in the colon of patients with PSC-UC (n=9) compared with UC alone (n=9), suggesting a potential T_H1 driven immune response within the PSC-UC mucosa.[84] However, the levels were similar to those in healthy controls, so again caution should be given to interpretation of these findings. They also found a higher

prevalence of innate lymphoid cells within the colon of patients with PSC-UC compared with UC, but these constituted less than 2% of lymphocytes, so the implication of these cells is unclear. They did not evaluate the presence of chemokine receptors or integrins on mucosal T-cells in PSC-IBD. No study to date has evaluated for the presence of CCR9+ or $\alpha 4\beta 7$+ T-cells in PSC-IBD as compared with IBD alone or healthy controls.

1.4 The immune system and gut-homing markers

1.4.1 Basic structure and function of the immune system

The human immune system is designed to combat the invasion of pathogens into the human body and maintain homeostasis. There are a number of levels to the immune system, starting from physical barriers such as the skin and mucus over intestinal epithelium, to the innate immune system which provides a rapid but non-specific response to invading pathogens, to the adaptive immune system. The adaptive immune system comprises both humoral and cell-mediated responses, the latter of which relies on the activation and response of T-cells and B-cells.[85]

There are two main types of T-cells: T-helper cells (CD4+) and cytotoxic T-cells (CD8+ T-cells). CD4+ T-cells recognise peptides/antigens from internally processed proteins/viruses which are presented in a complex with MHC-II on the surface of antigen presenting cells (APCs). Upon contact between the CD4+ TCR and the MHC-II-peptide complex, as well as a secondary costimulatory signal (B27-CD28), the CD4+ T-cell is activated and proliferates, becoming an effector T-cell. The role of the effector CD4+ T-cell, as per its name of “T-helper”, is to help other cells in their functions (which are often

proinflammatory and cytotoxic against pathogens). It does this by releasing various cytokines, activating B-cells, and promoting the function of various phagocytic cells such as macrophages, neutrophils and NK cells.[85]

Cytotoxic T-cells (CD8+) on the other hand, by virtue of their name, induce direct apoptosis of their target cells once activated. They are activated in a similar manner to CD4+ T-cells with two signals, however the initial signal is by interaction of its TCR with an MHC-I-antigen complex, by any cell of the body (usually a pathogen infected cell presenting a peptide antigen on the surface associated with MHC-1).

There are three main types of T-cells: naïve, effector, and memory. Naïve T-cells are those which have not encountered antigen and circulate mainly in the peripheral circulation. Upon encounter of the naïve T-cell with antigen and subsequent activation (which occurs in lymphoid tissue), the T-cell becomes an effector T-cell and carry out its various functions as mentioned above. Memory T-cells are long-lived T-cells which have previously been effector cells, but have survived the previously encountered infection/ inflammation, and are ready to have a rapid response and proliferate should the antigen be encountered again. Memory T-cells may be classified as effector memory or central memory, where the former still are able to carry out their effector functions.

Effector/ memory CD4+ T-cells can be of varying polarisations, such as T_H1, T_H2, T-regulatory (T_{reg}) and the more recently described T_H17 cells.[86] The destined phenotype of the cell depends on a unique set of transcription factors and the local cytokine environment in which the T-cells reside, and each subtype has different primary

functions. Briefly, interleukin-12 (IL-12) and transcription factor Tbet promote T_H1 differentiation (leading to cell-mediated immunity and tissue inflammation); IL-4, IL-25 and transcription factor GATA3 promote T_H2 differentiation (leading to enhanced humoral immunity, allergy and host defence against parasites); IL-6, TGFβ, IL-23 and transcription factor ROR-γ promote T_H17 differentiation (involved in autoimmunity as well as wound healing and host defence); and finally TGF-β and transcription factor FOXP3 promote T_{reg} differentiation (suppression of immune responses and homeostasis).

T-cells need to gain access to various tissue compartments to survey for pathogens and respond to pathogens encountered within that tissue. Usually, T-cells travel down the fast lane in the centre of blood vessels. For the lymphocytes to overcome these shear forces and slow down and subsequently enter the tissue, for example the intestinal lamina propria, various components both on the lymphocytes and on the endothelial cells must be present.[1] These include selectins and their ligands on the lymphocyte membrane, cytokines expressed by the endothelium (often upregulated in the setting of tissue injury/ inflammation) making contact with chemokine receptors on the T-cell, and finally mucosal addressins (adhesion molecules) on the endothelium interacting with integrins on the lymphocyte surface. All of these components may collectively be termed “gut-homing markers” in the setting of those which promote recruitment of lymphocytes to the bowel.

Chemokines are types of cytokines which cause chemoattraction towards a certain tissue (“chemoattractant cytokines”).[87] They can be expressed by virtually any cell type upon activation, including activated endothelial cells, epithelial cells and leukocytes

themselves. Once they are secreted, they are tethered to the endothelial surface by glycosaminoglycans, so that lymphocytes expressing the appropriate chemokine receptor can bind, become activated and trigger expression and activation of integrins. This is part of an "adhesion cascade" which brings about recruitment of lymphocytes to tissue compartments, which is discussed in more detail below.

1.4.2 CCR9 and CCL25

CC Chemokine Receptor 9 (CCR9) is a seven transmembrane G-protein coupled receptor which is expressed on the surface of leukocytes. Originally described in 1999, it is present on the vast majority of intraepithelial and lamina propria lymphocytes in the small intestine, and around 25% of lymphocytes in the colon expressing CCR9.[88-90] It has been shown to interact with its only identified ligand, chemokine (C-C motif) ligand 25 (CCL25) which is present in the thymus and small bowel, and, under inflammatory conditions, in the colon.[91]

CCL25 was originally named Thymus Expressed Chemokine (TECK) due to its identification within the thymus of recombinase activation gene-1 (RAG-1) deficient mice.[92] Interactions between CCR9 and CCL25 are thought to play a role in attracting progenitor cells to the thymus to aid development of mature $\alpha\beta$ and $\gamma\delta$ T-cells.[93] However, it has subsequently been identified that the interaction between CCR9 and CCL25 is a key part of T-cell recruitment to the bowel, particularly the small intestine.

In various murine and human studies, it has been shown that dendritic cells (DCs) derived from gut associated lymphoid tissue (GALT), but not splenic, hepatic DCs and stellate cells, can prime T-cells to express surface CCR9 and $\alpha4\beta7$.[94-97] GALT includes

mesenteric lymph nodes (draining the gut) and Peyer's Patches (lymphoid follicles located within the ileal mucosa).

Iwata *et al.* demonstrated that naïve T-cells are primed to express $\alpha4\beta7$ and CCR9 in response to T-cell stimulation in the presence of all-*trans*-retinoic acid (ATRA).[98] ATRA is a physiologically active metabolite of retinol (dietary Vitamin A). In this study, they stimulated naïve T-cells with anti-CD3 and anti-CD28, and cultured the cells with IL-2 in the presence or absence of 10nM ATRA. They demonstrated that T-cells exposed to ATRA (but not the negative control) significantly upregulated the expression of CCR9 and integrin, $\alpha4\beta7$. Furthermore, these cells migrated towards increasing CCL25 concentrations in a chemotaxis transwell assay. Finally, they showed that adoptive transfer of equal numbers of ATRA-exposed T-cells and non-ATRA exposed T-cells into non-transgenic mice led to a larger number of labelled T-cells in the small intestine in the ATRA group compared with the non-ATRA group. They also showed that Vitamin A deficient mice were deficient of T-cells within the small intestine.

Taken together, these findings indicate that priming of naïve T-cells to express $\alpha4\beta7$ and CCR9 occurs within GALT, relies on the presence of ATRA, and that these cells preferentially home to the small intestine. Subsequent studies have confirmed the chemotactic response to CCL25 of these cells.[99-101]

The majority of CCR9+ T-cells are memory cells (CD45RO+), and when stimulated are usually of the T_H1 phenotype, expressing IFN-γ though a small proportion produce IL-17

(T_H17 phenotype).[102-104] They are therefore inherently pro-inflammatory in nature. Indeed, they have been shown to be increased in various inflammatory settings.

In active small bowel CD and coeliac disease it has been demonstrated that there are elevated circulating CCR9+ CD4+ T-cells compared with healthy controls (8.1% versus 3.6% respectively).[105] This difference was not evident in patients with colonic CD. It has been shown separately that there is a subpopulation of $\gamma\delta$ T-cells which express CCR9, with a higher proportion of CCR9+ $\gamma\delta$ T-cells in the peripheral blood in UC compared with healthy controls.[106] Conversely, a group within our laboratory has shown that there is a reduced proportion of CCR9+ memory CD4+ T-cells in patients with UC as compared with healthy controls, but were unable to demonstrate a statistically significant difference between patients with PSC/UC and either UC or healthy controls.[84] They also showed a lower proportion of β7+ memory CD4+ T-cells in patients with PSC/UC and UC compared with healthy controls. β7, and in particular, the integrin $\alpha4\beta7$, is discussed further below.

Whilst historically CCL25 has not been identified in the large bowel under homeostatic conditions, colonic CCL25 has been identified in various murine models of colitis.[107-109] More recently, Trivedi *et al.* identified CCL25 in inflamed human colonic tissue from patients with UC via protein immunoprecipitation and Western Blotting as well as ELISA analysis of tissue protein lysates.[91] Furthermore, the expression of CCL25 was positively correlated with the endoscopic Mayo score. Hand in hand with this, they showed via flow cytometry that CCR9+ CD4+ and CD8+ T-cells were identified in abundance in the colon of patients with refractory UC (i.e. inflamed colon), with modest amounts in uninflamed colonic tissue from patients with active UC. Very few T-cells from healthy colonic tissue

were positive for CCR9. They also showed that in colonic CD, there was a high proportion of CD4+ and CD8+ T-cells which were CCR9+ in inflamed colon, with a lower proportion in matched uninflamed colon (n=2). Published around the same time, Bekker *et al.* actually did detect low levels of CCL25 in healthy colonic tissue, through Northern Blotting as well as immunohistochemistry (IHC). Similar to Trivedi *et al*, they found increased expression of CCL25 in the inflamed UC colon, as well as the inflamed CD colon.[109]

These findings are in contrast with earlier work by Papadakis *et* al. where there was a lower CCR9 expression in inflamed CD small bowel compared with adjacent uninflamed CD small bowel, as shown by both flow cytometry and Northern blotting (n=3).[105] They showed that *in vitro* activation of small bowel lamina propria lymphocytes with phytohaemagglutinin (PHA) led to a lower proportion of CCR9+ T-cells as compared with no stimulation. Furthermore, there was a higher proportion of CCR9+ T-cells which were dead or displayed an apoptosis marker (Annexin V) compared with CCR9- T-cells, indicating potential preferential activation-induced cell death of CCR9+ T-cells.

Finally, a small molecule antagonist of CCR9, Vercinon, has been studied in Phase II and Phase III clinical trials in CD. Whilst initial Phase II studies showed some promise for a benefit with regards to clinical response, subsequent Phase III studies were negative leading to the termination of the drug development programme.[110, 111] Interestingly, despite the relative abundance of CCR9+ T-cells in the small bowel as opposed to the large bowel, the subpopulation of patients with colonic CD (but not small bowel CD) had a higher proportion of clinical responders in the Vercinon twice daily group compared with the placebo group. This finding must be interpreted with caution, however, as it was a

secondary analysis, the numbers were small (n=54-66 in each group), and the placebo rate was unusually low in this group (13%).

Overall, the data demonstrate that the CCR9-CCL25 pathway is integral to the recruitment of T-cells to the intestine, and that this pathway is likely implicated in the pathology of inflammation in CD and UC.

1.4.3 α4β7 and MAdCAM-1

The integrin α4β7 has also been identified as a gut-tropic marker on mononuclear cells.[112] It was originally described in 1984 by Lazarovits *et al*. and known as "ACT-1".[113] In its original development, it was thought to be a lymphocyte activation marker and it was identified as a potential therapeutic target in preventing kidney transplant rejection. Subsequently, it was identified that ACT-1/α4β7 was an integrin expressed on human T-cells which had the hallmarks of gut tropism, including its ability to bind to adhesion molecule, Mucosal Addressin Cellular Adhesion Molecule-1 (MAdCAM-1).[112, 114] α4β7 has two other ligands to which it can bind: fibronectin and vascular cell adhesion molecule-1 (VCAM-1). However, binding of α4β7 to MAdCAM-1, after triggering by the CCR9-CCL25 interaction, is thought to be the principle mechanism for intestinal endothelial adhesion and subsequent migration of lymphocytes to the gut.

In human peripheral blood, as detected by flow cytometry binding with vedolizumab (VDZ), α4β7 is present on the majority of CD4+ and CD8+ T lymphocytes and B lymphocytes, though mainly at intermediate levels.[115, 116] It is also present on a proportion of natural killer cells, eosinophils and basophils at low to intermediate

levels.[115-118] Up to 15% of circulating monocytes express low levels of $\alpha4\beta7$, and it is not found on neutrophils.[116] With regards to T-cells, the highest expression of $\alpha4\beta7$ is seen on memory CD4+ T-cells - around 25% express high levels, with a further 5-10% expressing intermediate levels.[116] The relative expression of $\alpha4\beta7$ on various leukocyte subsets is illustrated in *Figure 1-1* and *Table 1.1*.

Whilst naïve T-cells express $\alpha4\beta7$, it is important to note that $\alpha4\beta7$ is not able to bind to its ligand, MAdCAM-1, until it is activated, usually by the binding of chemokine receptor, CCR9, to its ligand, chemokine, CCL25.[2] Therefore, the majority of $\alpha4\beta7$ within the peripheral circulation is not functionally active. Furthermore, it is felt that $\alpha4\beta7$ is present on many T-cells at birth, with higher amounts of T-cells positive for $\alpha4\beta7$ in neonates as compared with in adults (see *Table 1.1*).

The distribution of $\alpha4\beta7$+ mononuclear cells in the human body has been shown to be confined to circulating peripheral blood, lymphoid tissues and the gastrointestinal tract.[116] Through immunohistochemical detection of $\alpha4\beta7$ in a large array of healthy human tissues, the highest concentration of $\alpha4\beta7$+ mononuclear cells was seen in the large and small intestine, and in particular they were identified in submucosal lymphoid follicles and scattered through the lamina propria. In this particular study, there were no $\alpha4\beta7$+ cells seen in the healthy human liver.[116]

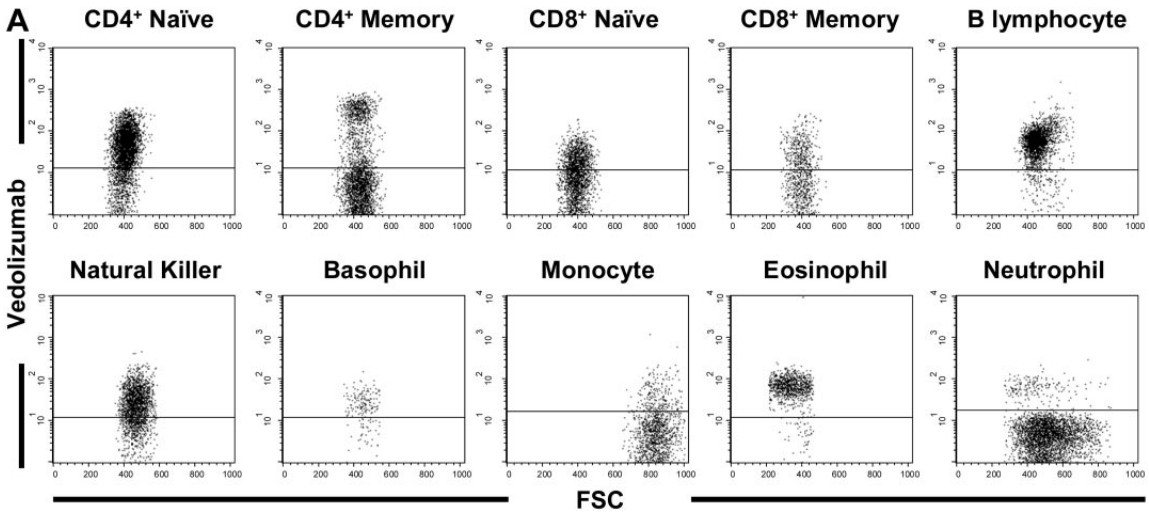


Figure 1-1 - Expression of $\alpha 4\beta 7$ across various leukocyte subsets

The expression of $\alpha 4\beta 7$ on various subsets of leukocytes is shown in these representative flow cytometry scatterplots on peripheral blood from a healthy human donor. Fluorescently labelled vedolizumab has been used to identify $\alpha 4\beta 7$+ cells on the Y-axis, with cells which are positive for $\alpha 4\beta 7$ above the horizontal line. Horizontal line determined from a negative control antibody. Each scatter plot represents a subpopulation of cells as identified by appropriate cellular markers. CD4+ memory cells have the highest expression of $\alpha 4\beta 7$, and CD4+ naïve cells and CD8+ cells have subsets with intermediate expression of $\alpha 4\beta 7$. Figure reproduced with permission from Soler *et al*.[116]

Table 1.1 - Proportion of various immune cells expressing $\alpha 4\beta 7$ in peripheral blood and relative expression per cell.

	CD3+ T-cells		CD4+ T-cells		CD8+ T-cells		B-cells	Monocytes	Eosinophils	NK cells	Neutrophils
	CD4+	**CD8+**	**Naïve**	**Memory**	**Naïve**	**Memory**					
Adults	25-60%	40-80%	>50%	~40%	50-90%	20-60%	40-70%	~15%	~100%	<50%	0%
Neonates	60-80%	~90%	-	-	-	-	-	-	-	-	-

NK, natural killer. Colour codes: shown below, reflective of the mean fluorescence intensity on $\alpha 4\beta 7$+ cells as a surrogate marker for receptor expression. Data adapted from references [115, 116, 119].

Expression level code:	Low	Low to intermediate	Intermediate	Intermediate to high	High

$\alpha4\beta7$+ cells have been identified via IHC in the PSC liver. This was seen in a study from 1999, examining liver biopsies from patients with PSC, PBC, and non-inflamed liver controls. 7/9 patients with PSC had greater than 10-50% portal mononuclear cells staining positive for $\alpha4\beta7$.[12] In contrast, virtually no $\alpha4\beta7$ was seen in the non-inflamed liver controls (1/7 has <10% positive for $\alpha4\beta7$, 6/7 had 0%), and low levels were seen in a proportion of livers affected by primary biliary cholangitis (PBC).

The locations of $\alpha4\beta7$+ cells described above correlate with the locations of its ligand, MAdCAM-1, which has been shown to be present in human lymphoid and intestinal tissue on immunohistochemical analysis.[120] In the small and large intestine, it is expressed constitutively on the venular endothelium of the lamina propria and submucosa, as well as intense expression on the endothelial lining of high endothelial venules within Peyer's patches and mesenteric lymph nodes. Its expression in the ileum and colon is increased in the setting of IBD (both CD and UC), particularly in areas where there are inflammatory infiltrates, indicating a potential association of MAdCAM-1 in the pathology of IBD.[120]

Whilst MAdCAM-1 has not been identified in the healthy human liver, subsequent studies have shown its presence in the diseased liver, similar to $\alpha4\beta7$. Grant *et al.* demonstrated immunohistochemical staining for MAdCAM-1 in diseased liver, but none in healthy liver.[121] Furthermore, a higher proportion of patients had hepatic MAdCAM-1 identified if they had PSC or autoimmune hepatitis as opposed to other liver diseases (such as primary biliary cholangitis, alcoholic liver disease, and chronic rejection post liver transplantation). Subsequently, the expression of MAdCAM-1 at a

transcriptional level has been demonstrated to be upregulated in the diseased human liver as compared with minimal to no expression in the healthy liver, with the highest levels in PSC.[122] . Additionally, it was shown through tissue-based assays that $\alpha4\beta7$+ T-cells can bind to hepatic MAdCAM-1 through the action of chemokine, CCL25, which can be blocked with antibodies against $\alpha4\beta7$ and MAdCAM-1.

1.4.4 CD161 and MAIT cells

Mucosal-associated invariant T-cells (MAITs) are a recently described subset of T-cells which possess both innate and adaptive immune properties.[123] They have a high expression of the CD161 receptor, which was originally identified as a C-type lectin-like receptor seen on the majority of natural killer (NK) cells. They are also characterised by a semi-invariant T-cell receptor (Vα7.2-Jα33/12/20) which recognises a restricted set of riboflavin precursors (bacterial metabolites) as presented by the MHC-like protein 1 (MR1). Whilst they can be activated traditionally through their TCR, they are also capable of innate stimulation by pro-inflammatory cytokines such as IL-12 and IL-18, leading to the expression of pro-inflammatory cytokines such as IFNγ and IL-17 or cytotoxic granzymes, such as Granzyme B and perforin.

MAIT cells are usually CD8+ T-cells, though occasionally are CD4+ or double negative T-cells, and make up around 2-8% of the peripheral blood T-cell population.[124] They are abundant in the healthy human bowel and liver, hence their original notation as mucosal-associated T-cells.

There are a proportion of CD4+ and CD8+ T-cells which express CD161 without having the Vα7.2 TCR of MAIT cells, which make up 20-30% of human T-cells and there is also a subset of CD161+ $\gamma\delta$ T-cells.[125, 126] These cells are also abundant in the bowel relative to the blood. Furthermore, these cells (particularly those with high CD161 expression) share similar functional and transcriptional properties, including the ability to secrete IFN-γ in response to IL-12/IL-18 stimulation.[126, 127, 123] Hence, when evaluating the role of these cells at mucosal sites, such as within the intestine and liver, it is important to not only analyse MAIT cells, but also the entire 161+ population. For the purposes of this thesis, cells with high CD161 expression are denoted as CD161++ and those with intermediate CD161 expression are denoted as CD161+mid. The term CD161+ denotes the total of both the CD161++ and CD161+mid population.

Several studies have shown that MAIT cells are depleted in the peripheral blood of patients with IBD (both UC and CD) as compared with healthy controls (HC).[128-130] This has also been shown to be true in PSC/IBD compared with HC.[131, 132] This is unlikely to be a specific feature of IBD and PSC, as depletion of MAIT cells has also been shown in other chronic diseases such as human immunodeficiency virus (HIV) infection, chronic hepatitis C, Dengue fever and severe influenza.[133]

In UC, a higher proportion of peripheral MAIT cells secrete IL-17 in response to *in vitro* stimulation with Phorbol 12-myristate 13-acetate (PMA) and ionomycin compared with HC.[130] It has also been shown that a higher proportion of MAIT cells express activated caspases in IBD compared with HC, possibly indicating increased apoptosis of

MAIT cells in IBD.[129] A recent study on MAIT cells in PSC/IBD showed that peripheral MAIT cells had higher PD-1 and CD38 expression in PSC/IBD compared with HC, as well as an impaired response to *E. Coli* or innate stimulation.[131] These data might indicate an activated exhausted phenotype of peripheral MAIT cells in PSC/IBD.

Data regarding the role of MAIT cells within the intestine in IBD are conflicting. In one study of patients with Crohn's disease, there was an increased proportion of MAIT cells (expressed as a percentage of total CD3+ T-cells) in injured ileum compared with adjacent healthy ileum in patients with Crohn's disease (n=11).[128] This was evaluated by confocal microscopy and triple staining immunofluorescence, though the antigens identified – CD3+, Vα7.2+ and IL-18Rα allowed for the possibility of either missing or over-estimating MAIT cells, due to the substitution of IL-18Rα for CD161. A subsequent study using the same confocal immunostaining technique, identified a higher proportion of MAIT cells in the colons of patients with active UC (n=5) as compared with inactive UC (n=7).[130]

Conversely, Hiejima *et al.* found reduced MAIT cell proportions among inflamed UC colonic tissue and inflamed CD ileum as compared with healthy colon and healthy ileum respectively.[129] They evaluated this via IHC and flow cytometry, in both cases identifying MAITs as positive for both CD161 and Vα7.2. Our own lab has identified lower proportions of MAITs and CD4+ 161+ T-cells in the inflamed intestine in IBD (both ileum and colon, mixed) as compared with HC via flow cytometry, again utilising the accepted method of CD161 and Vα7.2 identification of MAIT cells (T. Leng, *unpublished*).

Finally, there have been a variety of publications indicating reduced MAIT cell frequency in the liver in various chronic liver diseases including alcoholic liver disease, autoimmune liver disease, and chronic hepatitis C.[132, 134-136] Immunohistochemical analysis showed MAIT cells to particularly congregate around the bile ducts within the portal tracts. Whilst some of these studies included patients with PSC, they were analysed together with patients with other autoimmune diseases so clear inferences on liver MAIT cells in PSC cannot be conclusively drawn. There is no published literature on CD4+ and CD8+ 161+ cells in the bowel or the liver in IBD or PSC (as opposed to the subset of MAIT cells).

1.5 Implications for a role of gut-homing lymphocytes in PSC/IBD

Given the strong relationship between PSC and IBD, it is not surprising that there is evidence for a link between immune cells originating from the gut being involved in the pathophysiology of PSC.

The first evidence for these gut-tropic lymphocytes being implicated in PSC came from a human translational study from Birmingham, UK.[121] Grant *et al.* demonstrated that MADCAM-1 was also expressed in the liver, and was particularly localised to the portal veins , as identified by IHC. They showed that there was a greater hepatic expression of MAdCAM-1 in PSC (and autoimmune hepatitis) relative to other chronic liver diseases. Furthermore, they showed through tissue-based assays that $\alpha 4\beta 7$+ T-

cells can bind to hepatic MAdCAM-1 through the action of CCL25, which can be blocked with antibodies against $\alpha4\beta7$ and MAdCAM-1.

The group then demonstrated that there was a significant population of gut-phenotypic T-cells within the human liver in PSC as compared with other liver diseases or healthy liver donors.[137] They demonstrated via flow cytometry that 20% of PSC liver infiltrating T-cells were positive for CCR9 (most of which were also positive for $\alpha4\beta7$) compared with less than 2% of control liver-infiltrating T-cells. Furthermore, there was strong expression of CCL25 in the PSC liver compared with controls, as demonstrated by Western blotting and quantitative PCR. This higher expression of these gut-derived T-cells present in the PSC liver was validated in a study some ten years later by Liaskou *et al*.[138]

The question of whether these gut-phenotypic T-cells within the PSC liver are derived from the gut or actually primed with CCR9 and $\alpha4\beta7$ within the liver (and hence, did not come from the gut at all) was answered in a further study by Eksteen *et al.*[97] They co-cultured naïve CD8+ T-cells with hepatic dendritic cells (DCs), portal lymph node DCs, stellate cells, or intestinal DCs. Only those cultured with intestinal DCs induced high levels of expression of CCR9 and $\alpha4\beta7$, and there was minimal to no effect with the hepatic/portal derived cells. These studies included cells derived from human PSC livers. Hence, it was concluded that the CCR9+ $\alpha4\beta7$+ T-cells seen within the PSC-liver have their origins from the intestine.

Subsequently a collaborative study was performed between the UK (Birmingham) and the Norwegian PSC Research Center.[139] They carried out high-throughput sequencing of T-cell receptor β repertoires from matched colon, liver and blood samples from patients with PSC/IBD and normal gut and liver samples from colon cancer patients. There was a significantly higher overlap between the gut and liver of T-cell clones from PSC/IBD patients (mean 8.7%) compared with controls (3.6%). This difference must be borne against the fact that the PSC/IBD tissue was fresh-frozen tissue compared with the control tissue which was formalin-fixed, paraffin-embedded (FFPE). Whether the formalin and/or paraffin had any bearing on breakdown of T-cells or their receptors which could confound the finding is unclear. Nevertheless, the implication is that there are more shared characteristics between gut and liver T-cells in PSC/IBD compared with so-called healthy controls, lending further support for a role of gut-derived T-cells in the pathogenesis of PSC.

Lastly, a role for a related molecule, vascular adhesion protein-1 (VAP-1), has been put forward.[140] VAP-1 is constitutively expressed on sinusoidal endothelial cells in the liver, and, through its enzymatic activity, can assist with lymphocyte recruitment via induction of MAdCAM-1 expression by endothelial cells.[141, 142, 122] Hepatic VAP-1 expression has been shown to be greater in diseased liver as compared with healthy liver, and to the greatest extent in PSC.[140] Furthermore, it has been shown that VAP-1 enzymatic activity is elevated in PSC versus normal liver, and that this activity promotes adhesion of $\alpha 4\beta 7$+ lymphocytes to the hepatic endothelium.[140] VAP-1 exists also in a soluble form (sVAP-1) , and not only have higher sVAP-1 levels been reported in PSC versus controls, but within PSC patients, a higher sVAP-1 level is

associated with a worse clinical outcome (that is, reduced transplant-free survival).[140]

Multiple biologic drugs exist which are capable of therapeutically targeting this pathway and may be exploited for this purpose in PSC. A phase II open-label trial is currently underway in UK evaluating the efficacy of a monoclonal antibody, timolimumab, against VAP-1 in PSC.[143] There is also a licenced monoclonal antibody against $\alpha4\beta7$, vedolizumab (discussed further below). A cartoon illustrating these gut-homing molecules and lymphocytes in the setting of the PSC hepatic sinusoid, and the respective potential targeting monoclonal antibodies, is shown in *Figure 1-2*.

Figure 1-2– (next page) Diagram illustrating the presence of various gut-homing molecules on cells within the PSC liver and potential targeting biological therapy

The image depicts a hepatic sinusoid, where gut-tropic T-cells are slowed down and adhere to the sinusoidal endothelium through the interaction between CCR9 and its cognate ligand, CCL25, as well as binding of $\alpha4\beta7$ integrin to MAdCAM-1. VAP-1 also promotes lymphocyte recruitment through multiple mechanisms, including induction of MAdCAM-1 expression. Two monoclonal antibodies are depicted as targeting antigens and interrupting these pathways – vedolizumab ($\alpha4\beta7$) and timolimumab (VAP-1). CCL25, CC ligand 25; CCR9, C-chemokine receptor 9; LSEC, liver sinusoidal endothelial cells; MAdCAM-1, mucosal addressin cellular adhesion protein-1; PSC, primary sclerosing cholangitis; TMO timolimumab; VAP-1, vascular adhesion molecule-1; VDZ, vedolizumab. I acknowledge Servier Medical Art (https://smart.servier.com) for providing the use of some images in the creation this figure.

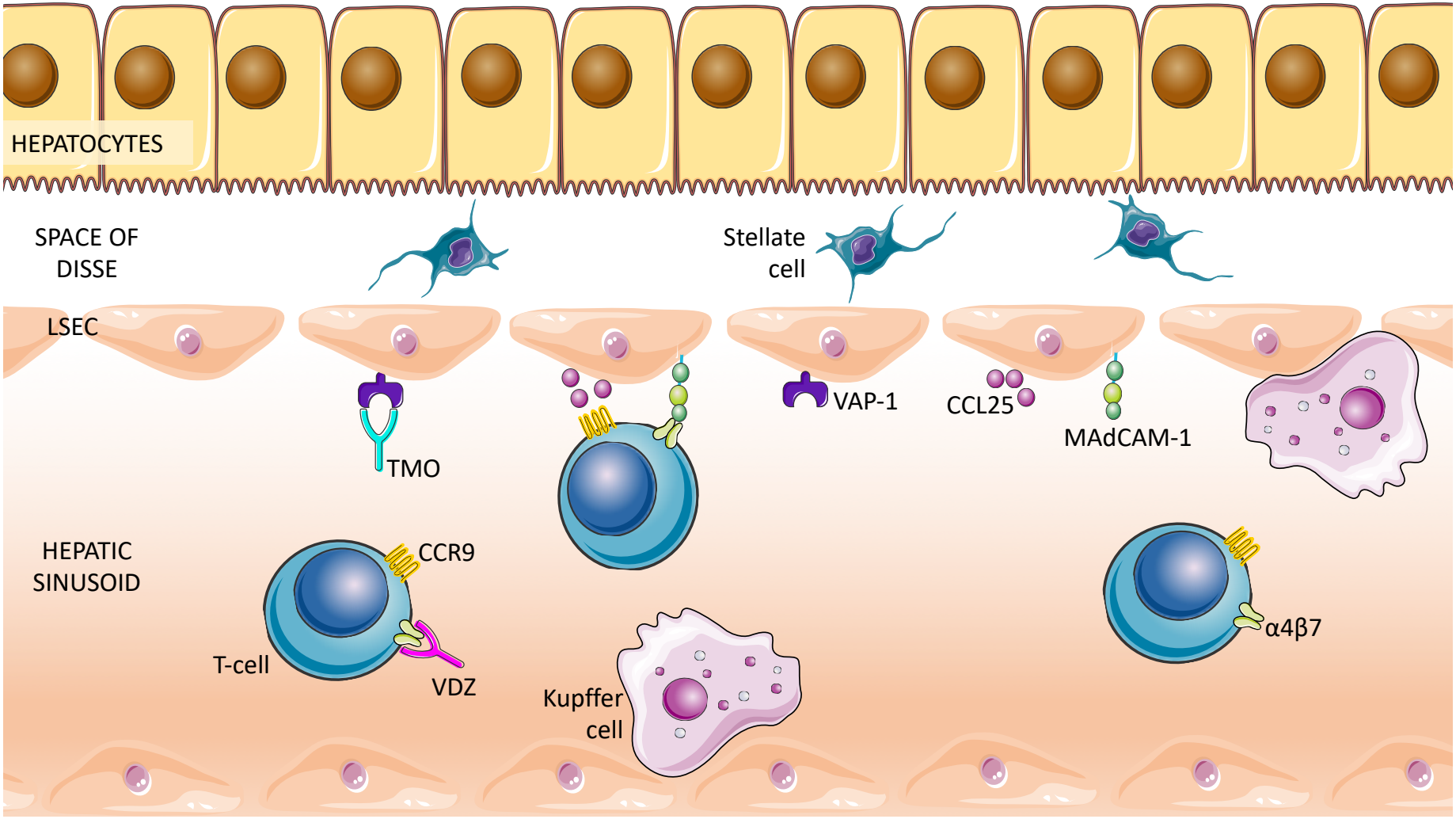


Figure 1-2 - Diagram illustrating the presence of various gut-homing molecules on cells within the PSC liver and potential targeting biological therapy.

1.6 Vedolizumab

VDZ is a monoclonal antibody which is directed against $\alpha4\beta7$ and is licenced for use in inflammatory bowel disease (IBD). It is a humanised form of anti-ACT-1, a murine antibody against $\alpha4\beta7$ first described in 1984 by Lazarovits *et al*.[113] Its mode of action has been shown that by binding to $\alpha4\beta7$ on lymphocytes it prevents attachment of $\alpha4\beta7$ to its ligand MAdCAM-1, without inducing apoptosis or antibody-dependent or complement-dependent cytotoxicity.[144] This is thought to therefore inhibit recruitment of lymphocytes to the intestine, particularly in the setting of inflammatory bowel disease, though this actual inhibition of entry of $\alpha4\beta7$ cells to the gut has not been conclusively shown experimentally.

1.6.1 Role of VDZ in IBD

VDZ has been shown to be beneficial in both CD and UC, firstly through animal studies, then human Phase II and III randomised, double-blind, placebo-controlled trials, and subsequently with real world data. It has been known interchangeably as MLN0002, MLN02 and LDP-02, until it was named vedolizumab just prior to its licensing in 2014.

The GEMINI I (UC), II and III (CD) trials were the seminal phase 3 studies establishing the efficacy of VDZ in IBD.[145-147] The optimal dosing regimen determined for both UC and CD was an induction schedule of 300mg intravenous infusion at week 0, 2 and 6, and the subsequent maintenance dosing was 300mg intravenously 8-weekly.

Both studies had predominantly symptom-based primary endpoints with the harder endpoints of endoscopic response and remission designated as secondary outcomes. As indicated earlier, more recent studies have indicated a poor correlation between clinical symptoms and endoscopic and histologic response,[48] with current clinical trials in IBD requiring the latter as a primary endpoint.

Nevertheless, at the end of induction, a clinical response with VDZ was seen in 47.1% of patients with UC (vs. 25.5% in placebo arm), with 41.8% in clinical remission at 52 weeks (vs. 15.9% in placebo cohort).[145] The response and remission rates were slightly lower in the CD studies with a dual primary endpoint for the induction phase: 14.5% achieved clinical remission (vs. 6.8%) and 31.4% achieved a drop in Crohn's Disease Activity Index (CDAI) of 100 or more (CDAI-100 response, vs. 25.7%, p=ns).[146] However, at week 52, 39% of patients were in clinical remission (vs. 21.6%), a proportion similar to the remission rates seen in UC.

1.6.2 Evidence for a potential role for VDZ in PSC

As mentioned previously, there are several studies which suggest that the particular gut-homing pathway that VDZ targets is implicated in the pathophysiology of PSC.[121, 137, 148, 149, 12] This includes overexpression of MAdCAM-1 in the PSC hepatic endothelial cells, as well as related adhesion molecules and chemokine ligands which can promote $\alpha4\beta7$-MAdCAM-1 interactions, such as VAP-1 and CCL25 respectively.[137, 122] Therefore, together with the common coexistence of IBD with PSC, it is possible that VDZ may play a role in reducing lymphocyte infiltration into the liver in patients with PSC and thereby reducing hepatic and biliary inflammation.

1.6.3 Clinical reports of VDZ use in PSC/IBD

There are 5 published case reports/series,[41, 150-153] and 2 case series in abstract form only,[154, 155] which evaluate the effect of VDZ in PSC/IBD, comprising a total of 140 patients (see *Table 1.2*). These studies vary with regards to what endpoints they report on. Some are purely descriptive, many evaluate the liver biochemistry at various time-points, particularly the ALP, and some report the effect of VDZ on the IBD itself.

The two largest published case series come from the Mayo Clinic in the USA (n=27)[41] and a North American/ Australian collaboration (n=34).[153] It is important to note here that whilst 27 and 34 patients were included in these analyses respectively, liver biochemistry parameters and other endpoints were only available among a subset of these patients. Both studies evaluated ALP and other liver biochemistry parameters at baseline and two later time-points (month 6-8 and month 12-14 for one study, and week 14 and week 30 for the other). When evaluating the mean/median ALP in the overall cohort, there was no significant change in ALP, transaminases or bilirubin from baseline to any time-point in either study.

In one study, they split patients up into two cohorts – those with elevated baseline ALP (n=18) and those with normal baseline ALP (n=8).[153] In the first subgroup (raised ALP at baseline) there was a statistically significant drop in median ALP from baseline (475IU/L) to week 14 (322.5IU/L, p=0.025) and a further numerical non-significant drop at week 30 to 283IU/L (p=0.267).

Table 1.2 - Characteristics of studies of vedolizumab in PSC/IBD

Study	Year	Location	Design	*n*	Liver biochemistry improvement	Comments
Lim *et al*.[150]	2016	London, UK	Case series	10[b]	Unknown	Included patients with AISC and PSC. 4/10 (40%) had clinical response of IBD. Liver biochemistry was not formally evaluated.
Westerveld *et al.*[151]	2017	Florida, USA	Case report	1	Yes	ALP improved from 225 to 127 at 13 months on VDZ. Transaminases also improved. Inflammation and biliary stricturing improved on MRCP.
Coletta *et al.*[152]	2017	Milan, Italy	Case report	1	No	Patient had PSC/UC with ileal pouch anal anastomosis. Clinical and endoscopic remission of pouchitis. Correlated with increase in circulating $\alpha4\beta7$+memory CD4+ T-cells.
Christensen *et al.* [153, 156]	2018	Chicago, Wisconsin, & Michigan, USA & Melbourne, Australia	Case series	34[a,b]	Mixed response	Overall (ALP available for n=26), no significant change in ALP from baseline (268 IU/L) to week 30 (249 IU/L, p=0.99). In patients with ALP>ULN at baseline, ALP reduction at week 14 but not week 30 – may be confounded by concomitant UDCA. Clinical remission of IBD at week 30 in 55% for CD, 29% for UC. 1/28 (3.6%) pre-LT patients required LT while on VDZ. 2/34 (5.8%) developed cholangitis within 6 months VDZ.

Tse *et al.* [41]	2018	Minnesota, USA	Case series	27[a]	No	Non-significant rise of baseline ALP from 260 IU/L to 310 IU/L at month 6-8 (p=0.11) and 319 IU/L at month 12-14 (p=0.24). No change in transaminases. No change in biliary structuring/ dilatation on imaging.
Caron, *et al*.[154]	2018	22 centres in Belgium and France	Abstract: Case series	54	Yes in small proportion	Primary outcome was decrease of serum ALP by ≥50% from baseline to week 30. 4/54 (7.4%) reached primary endpoint at week 30. 3/37 (9.1%) achieved ≥50% ALP reduction at week 54. Overall, mean ALP level increased by 0.4xULN at week 54. No change in transaminases observed. 5/54 (9.2%) developed cancer (3 x CRC, 2 x CCA).
Doherty, *et al*.[155]	2018	Dublin, Ireland	Abstract: Case series	13[b]	No	Statistically significant rise at several timepoints in median ALP from week 0 (126 IU/L) through to week 36 (190 IU/L, p=0.028). Showed no significant ALP rise in IBD alone cohort (i.e. no PSC) on VDZ (n=31). Median faecal calprotectin improved from baseline to month 6.

[#]Note – only trials involving patients who had not undergone liver transplantation were included. [a]Note - ALP changes were evaluated on a subgroup of these numbers according to availability of biochemistry. [b]This is the total number of patients analysed, a portion of which included some patients receiving VDZ post liver transplantation.

ADA, adalimumab; AISC, autoimmune sclerosing cholangitis; ALP, alkaline phosphatase; CCA, cholangiocarcinoma, CD, Crohn's disease; CRC, colorectal cancer; IBD, inflammatory bowel disease; IFX, infliximab; LT, liver transplantation; IU/L, international units per litre; MRCP, magnetic resonance cholangiography; PSC, primary sclerosing cholangitis; PC, placebo controlled; *n*, number; RCT, randomized controlled trial; UC, ulcerative colitis; UCEIS, ulcerative colitis endoscopic index of severity; UDCA, ursodeoxycholic acid; ULN, upper limit of normal; VDZ, vedolizumab.

This suggests that 11/18 (69%) patients showed an improvement in ALP on VDZ although none completely normalised their ALP. Conversely, among those patients with normal baseline ALP, half (4/8) had a rise in their ALP, with an overall small but statistically significant rise in median ALP.

Whilst currently not in peer-reviewed manuscript form, the published abstracts offer further interesting possibilities of whether VDZ may be effective in PSC and whether there are certain subgroups which stand to benefit more.[157, 154, 155]

The other notable moderately large cohort to mention was published in abstract form very recently – the French *Groupe d'Etude Thérapeutique des Affections Inflammatoires du tube Digestif* (GETAID) cohort[154] which analysed 54 patients.[158] The primary endpoint was very strict: ALP reduction by ≥50% from baseline. 7.4% achieved this by week 30, and 9.1% by week 54. Some might argue that a smaller drop is clinically significant, or potentially a decrease below a certain threshold, such as <1.5xULN, which has been shown to be associated with a better clinical prognosis.[159, 160, 30, 161]

Finally it is important to point out that in four out of the seven studies/abstracts mentioned, the response of the IBD in PSC to VDZ was reported. In all of these cases there was a signal that a similar proportion of patients responded as to those with IBD alone, whether it be analysed clinically, by faecal calprotectin levels or endoscopically.

1.6.4 Pharmacokinetics of vedolizumab

VDZ is administered intravenously at a dose of 300mg at week 0, 2, and 6 for the induction phase, then every 8 weeks during maintenance phase. The dose does not need to be adjusted according to weight. The mean serum trough concentrations are 27μg/mL at week 6 and 11-13μg/mL at steady state during maintenance.[162]

Its volume of distribution is approximately 5L, and it does not cross the blood brain barrier. The serum half-life is 25 days, though the exact method of elimination is not fully understood. As with most monoclonal antibodies, because of its large molecular size, it is thought to be unable to be directly renally excreted, but rather, metabolised to smaller peptides and amino acids which can thereafter be excreted by the kidneys.[162]

The development of anti-drug antibodies is lower than other biologic agents such as anti-TNF agents and was at a rate of 4% in the GEMINI studies (56 of 1434 patients). A small proportion of patients (9 of 56) had persistently positive anti-drug antibodies and this correlated with lower or undetectable VDZ serum trough levels. Whilst this did not translate to a worse clinical outcome, the number of events was too small to make a definitive assessment.

1.6.5 Pharmacodynamics of vedolizumab

Upon administration of VDZ at the doses specified above, >95% of $\alpha4\beta7$ receptors are saturated, and in primate models, this has been demonstrated to occur within 30 minutes.[163, 164] VDZ is highly specific for $\alpha4\beta7$ and does not bind to $\alpha4\beta1$ nor $\alpha4E7$, and has been shown functionally *in vitro* to prevent binding of $\alpha4\beta7$ to MAdCAM-1 and

fibronectin but not VCAM-1.[116] Upon binding to $\alpha4\beta7$, it does not cause any cell death, nor does it engender any cytokine release.[144, 165] It has been shown that binding causes internalisation of the $\alpha4\beta7$ (*in vitro)* and, on withdrawal of the VDZ, $\alpha4\beta7$ is identified again at the cell surface within 24 hours and is functionally active with regards to MAdCAM-1 adhesion.[144, 165]

1.6.6 Alterations to circulating $\alpha4\beta7$+ cells in blood with vedolizumab

Until the last year or two, the data relating to the effect of vedolizumab on circulating $\alpha4\beta7$+ cells was limited to animal data. In 2012, Millenium Pharmaceuticals, the pharmaceutical company who initially developed VDZ in pre-human and early phase human studies, published their experience of VDZ in cynomolgus monkeys.[164] Using flow cytometry, they showed that the proportion of peripheral blood memory CD4+ T-cells which had high expression of $\alpha4\beta7$ (as opposed to negative or intermediate expression) increased from baseline to post-VDZ: 15-22% of to 62-73%.

Subsequently, in the last two years, some human data has emerged on the changes to the expression of $\alpha4\beta7$ in peripheral blood upon administration of VDZ.[164, 117, 165-167] It is somewhat difficult to compare these data, as they all examine slightly different subpopulations of cells (e.g. total CD4+ cells versus memory CD4+ cells), and some examine $\alpha4\beta7$ expression as proportions and some analyse the mean fluorescence intensity as a surrogate marker for the per-cell expression of the $\alpha4\beta7$ receptor.

Fuchs *et al.* analysed 36 patients with IBD (approximately half CD, half UC). They showed that any changes VDZ had upon the proportion of peripheral blood CD4+ T-cells which

expressed $\alpha4\beta7$ were linearly correlated with the changes seen within the CD8+ population.[166] They expressed proportions as the percentage of CD4+ T-cells (or CD8+) compared with the baseline percentage (T1, i.e. prior to VDZ). Whilst not giving the numerical data on what the changes in fact were post VDZ, nor what the changes were at different time points post-VDZ, the majority of points on the graph indicated that the proportions post VDZ dropped from baseline. In other words, after VDZ, there were less CD4+ and CD8+ T-cells expressing $\alpha4\beta7$ (i.e. <100% of T1), with the range appearing to be approximately 35-100%.

A further study by the same group supported this finding, analysing the peripheral blood of 13 patients with IBD (subtype not specified).[165] They showed that the proportion of CD3+, CD4+ and CD8+ T-cells which expressed $\alpha4\beta7$ reduced with VDZ, from approximately 20-30% of total cells, to 5-10%, over 2 infusions. They postulated the reason for this is that the $\alpha4\beta7$ receptor internalised upon binding with VDZ. Whilst they used VDZ Ab to stain the peripheral blood mononuclear cells (PBMC), they performed separate in vitro assays after culturing PBMC with VDZ initially and showed that the VDZ did not interfere with the binding of the fluorescently labelled VDZ.[168]

An American group examined the MFI of $\alpha4$ and $\beta7$ individually on circulating naïve T-cells pre- versus post-VDZ in around 18 patients with IBD (mixed UC, CD, and IBD-unspecified).[117] They showed the MFI went down post-VDZ, which would be in keeping either with internalisation of the $\alpha4\beta7$ receptor, or reduced number of cells in the blood. However, they showed that amongst CD4+ T-effector memory lymphocytes (CD45RA-

CCR7-), the proportion which were $\alpha4\beta7$+ increased pre- versus post- VDZ and postulated that this would be due to preventing trafficking of these cells to the intestine.

Lastly, another group from America looked at soluble unbound $\alpha4\beta7$ in the peripheral circulation of 32 patients with UC before and after VDZ.[167] The correlation between soluble $\alpha4\beta7$ (s-$\alpha4\beta7$) and leukocyte membrane bound $\alpha4\beta7$ is unclear. Nevertheless, they showed a rise in s-$\alpha4\beta7$ from virtually none present in the serum at baseline (median 1.3ng/mL) to increased levels at various time points up to week 26 on VDZ (week 2: 14.1ng/mL, week 26: 9.9ng/mL).

Taken together, these mixed data suggest that the proportion of circulating total and/or naive CD3+, CD4+ and CD8+ T-cells which are $\alpha4\beta7$+ reduces with VDZ administration, however, among memory populations the data varies from no change, to increases in $\alpha4\beta7$+ populations. As there are more $\alpha4\beta7$ receptors on CD4+ memory T-cells than any other cell population, it makes sense that these are the possible preferential targets of VDZ, though whether only some $\alpha4\beta7$ receptors are internalised on binding with VDZ, and/or whether $\alpha4\beta7$ is shed on binding is not known.

These data examining peripheral blood mononuclear subsets are limited to 3 human studies, each involving less than 20 patients, so existing data is limited to draw definitive conclusions on the effect of VDZ on circulating $\alpha4\beta7$+ lymphocytes. Data described evaluating peripheral blood alterations with VDZ are described in *Table 1.3*.

1.6.7 Alterations to α4β7+ cells in tissue with vedolizumab

Akin to translational analysis of α4β7 expression in the peripheral blood, studies examining its expression within the tissue are limited. The initial animal data in cynomolgus monkeys suggested via IHC that there was a reduction in β7+ cells per crypt in the colon in monkeys treated with VDZ compared with vehicle.[164] Furthermore, the intensity of the staining was lower in monkeys treated with VDZ, suggesting not only a reduction in the infiltration of β7+ cells into the colon (presumably α4β7+ given the specificity of VDZ), but also a selectivity of VDZ for cells with high expression of α4β7.

These data are at odds with recent human data from Zeissig *et al*.[169] They too performed IHC on colonic samples, in this case from 8 human patients with UC or CD. They analysed the proportion of CD3+ cells in the lamina propria at week 14 of VDZ, and did not show any reduction from baseline, both as % of total lamina propria mononuclear cells (LPMC), nor mean count per high power field (HPF). Incidentally, on IHC, they did show a slight reduction T-cells among LPMC in patients receiving infliximab (IFX, n=7).

They also analysed LPMC isolated from colonic biopsies and performed multicolour flow cytometry on samples at baseline and after 14-weeks of IFX (n=9) or VDZ (n=8). Once again, they showed a small reduction in T-cells with IFX (expressed as the proportion of total cells), but not with VDZ, and also showed no change with subsets of T-cells: CD4+, CD8+, central memory T-cells and effector memory T-cells.

Table 1.3 - Studies evaluating changes to $\alpha4\beta7$ expression in peripheral circulation with vedolizumab administration

Author and year of study	n	Host	Population(s) evaluated	Finding
Fedyk ER, *et al.* 2012 [164]	8-12	Cynomolgus monkeys	Memory T-cells (CD45RA- CD4+)	%$\alpha4\beta7$+ increased with VDZ: 15-22% to 62-73%.
Fuchs F, *et al.* 2017 [166]	CD = 12 UC = 12	Humans (CD or UC)	CD4+ and CD8+ T-cells	%$\alpha4\beta7$+ post-VDZ correlated linearly between CD4+ and CD8+ T-cells Most populations post VDZ had lower %$\alpha4\beta7$+ compared with baseline (30-100% of baseline)
Rath T, *et al.* 2018 [165]	13	Humans (mixed IBD)	CD3+, CD4+, CD8+, CD19+, CD14+, CD56+	%$\alpha4\beta7$+ decreased in all populations from baseline after 1-2 infusions of VDZ
Boden EK, *et al*. 2018 [117, 170]	26[a] (19 for analysis)	Humans (mixed IBD)	Naïve CD4+ and CD8+ T-cells CD4+ T effector memory cells (CD45RA- CCR7-)	MFI of $\alpha4$ and $\beta7$ went down with VDZ on naïve CD4+ and CD8+ T-cells %$\alpha4\beta7$+ of CD4+ T_{EM} increased post-VDZ
Battat R, *et al*. 2018 [167]	32[a] (12-20 for individual time-points)	Humans (UC)	Soluble-$\alpha4\beta7$ in serum	s-$\alpha4\beta7$[b] increased from baseline through to week 26 VDZ - Baseline 1.3ng/mL - Week 2 14.1ng/mL - Week 6 11.4ng/mL - Week 14 12.7ng/mL - Week 26 9.9ng/mL

[a]Though this number of patients were recruited, analysis described was only performed on a subset of patients as indicated. [b]It is not clear whether mean or median levels are indicated here. CD, Crohn's disease; IBD, inflammatory bowel disease; MFI, mean fluorescence intensity; n, number; UC, ulcerative colitis; VDZ, vedolizumab.

Whilst they did not look at $\alpha 4\beta 7$+ cells directly, their data suggested that VDZ did not reduce the trafficking of T-cells into the colon. This is spite of the fact that around half of the patients were VDZ remitters (and therefore one would expect there to be a reduction in T-cells due to reduced inflammation). These data are in contrast with how VDZ is postulated to work – targeting $\alpha 4\beta 7$+ T-cells, and thereby augmenting their trafficking to the bowel. Nevertheless, the data are in small numbers (only 5 remitters) and warrant further investigation.

Another group looked at the effect of VDZ on T-cell trafficking to the gut through a complicated humanised mouse model.[171] The NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mouse strain was used which lacks murine T cells, B cells and NK cells. Furthermore, the mice were pre-administered dextran sodium sulphate (DSS) which induces a colitis and is often used to model human UC. Then, labelled human T-cells, either from healthy controls or patients with UC, were injected into the mouse's ileocolic artery via a median laparotomy. These T-cells had been pre-treated *in vitro* either with VDZ or vehicle. Subsequently the colon was exteriorised and the colonic mucosa was positioned on a glass transparent petri dish and images of the colon were taken with a confocal microscope. Colonic LPMC were also isolated to perform flow cytometry analysis. Comparisons were made between VDZ-treated cells and vehicle treated cells among the two adoptive T-cell transfers (i.e. from patients with UC and from control patients).

They showed that VDZ caused a reduction in CD4+ T-effector cells (CD4+ CD25-) and CD4+ T-regulatory cells (CD4+ CD25+) to the murine inflamed colon if T-cells from patients with UC were adoptively transferred, but not from VDZ-treated human control T-cells. These

findings were shown both with confocal *in vivo* microscopy and with flow cytometric expression (n=4). They repeated the study with the same methods, but using T-cells from patients with CD, but, unexpectedly, could not replicate findings seen with VDZ-treated UC T-cells.[172] That is, only a modest reduction in lymphocyte accumulation was seen in the murine inflamed lamina propria with VDZ-treated T-cells from patients with CD, but it did not reach statistical significance..

It is evident from these four studies that whether VDZ actually does inhibit the homing of T-cells to the lamina propria of the colon or not is unclear, with even further uncertainty upon effects of trafficking to the ileum. Given that T-cells are thought to be integral to the pathophysiology of IBD, the fact that VDZ does indeed have a clinical benefit in IBD, particularly colonic disease, and the likely affinity for VDZ to CD4+ memory cells, further research, is warranted, particularly within humans.

1.6.8 $\alpha 4\beta 7$ expression in blood and tissue as a marker of response to vedolizumab

Identifying biomarkers to predict response to VDZ in the setting of IBD is of increasing importance given there are numerous alternative therapeutic agents in IBD. Previous data suggests that the choice of the first biologic is important, as patients are less likely to respond to a second biologic than the first biologic agent they are given.[76, 77]

With regards to other therapeutic agents in IBD, some biomarkers have been identified which can predict response. With relation to Crohn's disease, high expression of the cytokine, Oncostatin M, within the inflamed colonic mucosa is associated with failure of anti-TNF therapy.[173] In a recent Phase II UC trial of etrolizumab, a monoclonal antibody against the integrin $\alpha E\beta 7$, patients with higher colonic expression of αE, both

transcriptionally and via IHC, were more likely to enter clinical remission with etrolizumab.[174] Established biomarkers for VDZ response currently are not in use.

There have been a few studies which have attempted to look at biomarkers for VDZ response and/or remission, predominantly in blood, with mixed results.[117, 165-167] All of the outcomes of response or remission have been wholly or partially symptom based, rather than the harder endpoint of purely endoscopic or histologic scores, which reflects inflammation and therefore response to therapy more accurately. This therefore creates an initial limitation on interpretation of findings. Furthermore, the results are somewhat at odds with each other.

Fuchs *et al.* identified that the baseline $\alpha4\beta7$ expression among peripheral CD4+ T-cells (expressed as percentage $\alpha4\beta7$+ of total CD4+ T-cells by flow cytometry) was *lower* in patients with UC/CD with a clinical response to VDZ as compared with non-responders.[166] Meanwhile, Boden *et al.* found that clinical responders to VDZ (either UC or CD) had a *higher* baseline $\alpha4\beta7$ expression on naïve CD4+ and CD8+ T-cells (as expressed by mean fluorescence intensity) compared with non-responders. This was also true for T-effector memory cells (T_{EM}, CD45RA- CCR7-) and terminal effector memory T-cells (T_{EMRA}, CD45RA+ CCR7-) but not central memory T-cells (T_{CM}, CD45RA- CCR7+).

Meanwhile, Battat *et al.* report a more rapid rise in s-$\alpha4\beta7$ in the blood in remitters compared with non-remitters (UC patients treated with VDZ). However, whilst the mean s-$\alpha4\beta7$ levels were different between remitters and non-remitters, all patients had rises

in s-$\alpha 4\beta 7$ upon VDZ therapy, and the spaghetti plots do not show a clear differentiation between the two, so this finding should be interpreted with caution.

Finally, Rath *et al* looked at biomarkers within colonic tissue. They were able to demonstrate a higher mean count per HPF of $\alpha 4\beta 7$+ cells on colonic biopsies at baseline in patients with IBD (UC or CD) treated with VDZ who were subsequently remitters compared with non-remitters.[165] They also showed a lower proportion of $\alpha 4\beta 7$+ T-cells in post-VDZ colonic biopsies in remitters compared with non-remitters (as demonstrated by flow cytometry on LPMC), thereby demonstrating a proof of concept for the drug mechanism (unlike some of the data described above in 1.6.7).

Once again, given the conflicting and limited data, further studies are required to definitively define biomarkers for VDZ response and remission. Ideally, these would have endoscopic and/or histological determinants for response/remission, as these more accurately reflect the immune and inflammatory response.

Furthermore, there is no data evaluating the response to VDZ within the peripheral blood, intestine and liver at a cellular level in patients with PSC/IBD. Given that $\alpha 4\beta 7$ expression may be altered in PSC/IBD, it is important to evaluate whether there are differences in T-cell trafficking with VDZ between PSC/IBD and IBD alone.

1.7 Hypotheses and Aims

Given the implications for a role for gut-homing lymphocytes in the pathophysiology of PSC-IBD, as well as the presence of a drug targeting this pathway with beneficial results in IBD and unestablished outcomes in PSC-IBD, the following hypotheses were made:

1. *There is a differing distribution throughout tissues (peripheral blood, intestine and liver) of gut-homing lymphocytes* in PSC-IBD versus controls.*
2. *Gut-homing lymphocytes* accumulate within the liver in PSC-IBD but not in control livers.*
3. *Vedolizumab causes a reduction of ALP in a significant subset of patients with PSC-IBD.*
4. *Vedolizumab alters the distribution of gut-homing lymphocytes* in IBD, by reducing their prevalence within the bowel, and potentially preventing their entry into the liver in PSC-IBD.*

*Gut-homing lymphocytes as denoted by markers of CCR9, $\alpha 4 \beta 7$ and CD161

In keeping with these hypotheses, the broad aims of this thesis were to:

1. *Determine the proportion of gut-homing lymphocytes in the peripheral blood, ileum and colon (both inflamed and uninflamed) in patients with PSC/IBD as compared with healthy controls and UC.*
2. *Establish a technique for sampling liver infiltrating lymphocytes for immunophenotyping*

3. *Determine the proportion of liver-infiltrating lymphocytes which are positive for gut-homing markers in PSC compared with diseased liver controls and healthy controls.*
4. *Establish the clinical effect of vedolizumab on patients with PSC-IBD as compared with IBD alone with respect to IBD response and PSC response.*
5. *Establish the redistribution of $\alpha 4\beta 7$+ T-cells within the various tissue compartments in patients undergoing VDZ therapy for IBD.*
6. *Identify biomarkers for clinical response or remission for patients with IBD and/or PSC receiving VDZ therapy.*

2 Methods and Materials

2.1 Ethics

All human samples taken and human clinical data reported in this thesis were done so in accordance with established clinical studies with the relevant ethical approval and in line with the Declaration of Helsinki and Good Clinical Practice guidelines.[175]

The studies under which these samples and data were collected were:

1. Gastrointestinal Illness in Oxford: prospective cohort for outcomes, treatment, predictors and biobanking.
    - Research Ethics Committee Reference (REC Ref): 16/YH/0247
    - *Subproject: "Liver and intestine-specific lymphocytes in Primary Sclerosing Cholangitis and Inflammatory Bowel Disease (Ref:15/SK/KW/PSC-IBD)."*
2. Inflammatory Bowel Disease in Oxford: prospective cohort for outcomes, treatment, and predictors.
    - REC Ref: 09/HO603/19
    - *Subproject: "Liver and intestine-specific lymphocytes in Primary Sclerosing Cholangitis and Inflammatory Bowel Disease (Ref:15/SK/KW/PSC-IBD)."*
3. Analysis of liver infiltrating lymphocytes in primary sclerosing cholangitis by fine needle aspiration of the liver.
    - Short title: "The PSC FNA Liver Study."
    - REC Ref: 17/SC/0290
4. Analysis of hepatic T-cell responses from liver biopsies.
    - REC Ref: 09/H0603/19

2.2 Materials

2.2.1 Cell isolation and cell culture materials

Dulbecco's phosphate buffered saline (D-PBS), foetal calf serum (FCS), 4-(2-hyroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Penicillin-Streptomycin, Roswell Park Memorial Institute-1640 (RPMI-1640) with L- glutamine, Ethylenediaminetetraacetic acid (EDTA) Bovine Serum Albumin (BSA), DNase-1, Trypsin-EDTA 1X solution, Trypan Blue Stain, All-*trans*-retinoic acid (ATRA), human Interleukin-2 (IL-2), Collagenase D, Dimethyl sulfoxide (DMSO): Sigma Aldrich (Gillingham, UK)

Ultra-LEAF™ Purified anti-human CD3 Antibody (clone OKT3), Ultra-LEAF™ Purified anti-human CD28 Antibody (clone CD28.2): Biolegend, distributed by Cambridge Bioscience (Cambridge, UK)

Lymphoprep™: Stemcell Technologies (Vancouver, Canada)

GentleMACS™ C tubes, GentleMACS™ dissociator, Pan T-cell isolation kit (human), MACS® Separation columns (size LS): Miltenyi Biotech (Woking, UK)

Radial Jaw™ 4 Single-Use Large Capacity Biopsy Forceps – 2.4mm x 240cm (M00513311): Boston Scientific (Hemel Hempstead, UK)

Quinke Spinal Needle, 22G: Beckton Dickinson, distributed by Care Shop (Manchester, UK).

Lidocaine 1%: distributed by Oxford Universities NHS Foundation Trust pharmacy department (Oxford, UK)

10X Red Blood Cell Lysis Buffer: eBioscience, San Diego, CA.

2.2.2 Flow cytometry

Human Fc Receptor (FcR) blocker: Miltenyi Biotech (Woking, UK)

Invitrogen™ eBioscience™ 1X Red Blood Cell (RBC) Lysis Buffer: Fisher Scientific (Loughborough, UK)

BD Cytofix fixation buffer, LSR II™ flow cytometer: BD Biosciences (Woking, UK)

2.2.3 10X Genomics and single cell RNA sequencing

10x Genomics Chromium Single Cell 3' v2 Reagent Kit, Chromium Single Cell 3' Library & Gel Bead Kit, 10x Genomics Chromium Single Cell Controller: 10X Genomics (San Francisco, USA)

twin.tec 96-Well PCR Plate: Eppendorf (Stevenage, UK)

Pierceable Foil Heat Seal, PX1 PCR Plate Sealer (Bio-Rad, Watford, UK)

TapeStation High Sensitivity D1000 ScreenTape, Bioanalyzer D5000 ScreenTape High Sensitivity chip: Agilent Technologies (Cheadle, UK)

KAPA DNA Quantification Kit: Roche (Pleasanton, US)

HiSeq 4000 sequencing platform: Illumina (San Diego, US)

Veriti™ 96-well thermal cycler, magnetic Dynabeads™ MyOne™ SILANE: ThermoFisher Scientific (Waltham, MA)

SPRIselect Reagent: Beckman Coulter (High Wycombe, UK)

2.2.4 Immunohistochemistry

Superfrost glass slides (Thermofisher Scientific, Waltham, MA)

Histo-clear: National Diagnostics, distributed by Scientific Laboratory Supplies (Nottingham, UK)

Absolute ethanol, Molecular Biology Grade: Fisher Bioreagents, distributed by Fisher Scientific (Loughborough, UK)

Trizma® Base, Human serum (from human male AB plasma, USA origin, sterile-filtered), goat serum, Hydrogen peroxide solution (30% w/w in H_20 containing stabiliser), Mayer's haematoxylin solution: Sigma Aldrich (Gillingham, UK)

ThermoShandon Sequenza™: ThermoShandon Ltd, (Cheshire, UK)

Polyclonal anti-human CCR9 – extracellular domain ab140791 (isotype rabbit IgG): Abcam (Cambridge, UK)

CD3 monoclonal antibody (clone F7.2.38, isotype mouse IgG1, κ), EnVision Detection System (Peroxidase/DAB, Rabbit/Mouse): Dako (Ely, UK)

Biotinylated Goat Anti-Rabbit IgG Antibody, Vectastain® Elite ABC Kit: Vector Laboratories, (Peterborough, UK)

Aquamount: Merck (Kenilworth, New Jersey)

Aperio Scanscope Turbo T2, Aperio ImageScope (version 12): Leica Biosystems (Milton Keynes, UK)

Image Processing and Analysis in Java (Image J, version ImageJ2): National Institutes of Health (NIH) and Laboratory for Optical and Computational Instrumentation (LOCI), (Wisconsin, US)

2.3 Isolation of mononuclear cells from different tissue compartments

2.3.1 Isolation of peripheral blood mononuclear cells (PBMC):

Whole blood was diluted 1:1 with D-PBS at room temperature. This was overlaid on the density gradient medium, Lymphoprep™, and centrifuged at 973g without brake at room temperature for 20 minutes. The mononuclear layer was then isolated and washed in D-PBS three times, before being finally suspended in cell staining buffer (2% BSA, 0.2% EDTA

in D-PBS). The viable mononuclear cell count was obtained using staining with Trypan Blue and counting manually with a Neuabauer haematocytometer.

2.3.2 Isolation of Lamina Propria Mononuclear Cells (LPMC) from intestinal tissue

8-10 biopsies were obtained with forceps at colonoscopy or flexible sigmoidoscopy from ileum, uninflamed colon and/or inflamed colon, and placed immediately in culture media (RPMI-1640 with L-glutamine, 10% FCS, 1% penicillin/streptomycin) on ice. Within 4 hours, samples were transferred into GentleMacs™ C Tubes, suspended in 5ml culture medium with Collagenase D 1.0mg/ml and DNase I 0.1mg/mL, and after a gentle perturbation cycle on the GentleMACS™ dissociator, they were placed in a 37°C shaking incubator for one hour. They were then homogenised via a high speed cycle on the GentleMACS™ dissociator before being passed through a 70μm filter and washed three times in culture media. Finally they were resuspended in cell staining buffer (as above), with cell count obtained by staining a small sample with Trypan Blue solution and counting on a Neubauer haematocytometer.

2.3.3 Isolation of liver infiltrating lymphocytes (LILs) via fine needle aspiration of the liver.

Ultrasound examination was used to identify a suitable position for the liver FNA. Usually this was in the right mid-axillary line, 1-2 intercostal spaces above the costal margin. The biopsy field was cleaned with iodine antiseptic solution and, using a very fine bore needle (25Ga), 2-3ml 2% lignocaine was infused just under skin at biopsy site. The lignocaine syringe needle was swapped to a 21Ga needle, and the needle was inserted at the same site into the liver and the remaining 7-8ml 2% lignocaine was inserted to anaesthetise in particular the liver capsule and the tract leading to it.

A 22 Ga spinal needle, with an internal stylet, was then inserted through the same needle tract to 2-3cm into the liver parenchyma from the capsule. The internal stylet was removed and a 10ml syringe filled with 2ml ice-cold sterile saline was attached. Cells were then aspirated into the saline using repeated gentle back pressure 5-10 times. The stylet was then reinserted back into the needle, and the needle was withdrawn 1-2cm (without withdrawing from the body) then reinserted back into the liver, just under the capsule, but orientated in a slightly different direction when passing into the liver. Aspiration was repeated with a fresh syringe containing 2ml ice-cold sterile saline, then the whole needle was withdrawn with the syringe attached. This process was repeated a second time (but without repositioning) resulting in three syringes containing aspirate via two needles. The contents of the syringe were emptied through the spinal needle into a 50mL conical tube containing 40mL ice cold FNA culture media (RPMI-1640 with L-glutamine, 10% FCS, 1% penicillin/streptomycin, 25mM HEPES). The aspirate was passed through the spinal needle because it was noted this significantly increased the yield of LILs, likely due to trapping of LILs within the needle.

(If a liver biopsy was to also be performed for clinical purposes, this was then immediately subsequently performed by the treating doctor as per standard clinical protocol, utilising the same anaesthetised needle tract.)

The FNA sample was then centrifuged (within one hour of being obtained) at 2000rpm for 10 minutes at 4°C, and the pellet was resuspended in cell-staining buffer (as described above, but supplemented with 25mM HEPES, and no EDTA).

(Please see Appendix 8.3 for method for obtaining LILs from liver biopsies and resections.)

2.4 Flow cytometry

Mononuclear cells isolated from peripheral blood, intestine, or liver were transferred to a 96-well V-bottomed plate (up to 5 x 10^6 per well). Except for LILs, cells were incubated for ten minutes at 4°C with a blocking buffer (1:100 Human FcR blocker - 1µL per 1x10^6 cells), then washed with cell staining buffer. They were incubated with conjugated primary antibodies for 20 minutes at 4°C, then washed twice (once for FNA samples). Fluorescence Minus One (FMO) samples were also created for important antigens of interest. Samples were then incubated with 1X RBC lysis buffer at room temperature for ten minutes before being washed once.

Subsequently, PBMC and LPMC (not LILs) were fixed with 4.2% formaldehyde at 4°C for 15 minutes (Antibody Panel 1 cells only – not LILs), then washed twice, resuspended in cell staining buffer, and kept at 4°C before acquisition on flow cytometer (LSR II, BD Biosciences), within 72 hours of staining. LILs did not undergo fixation and were acquired on the flow cytometer immediately.

Cells stained with Panel 1 were acquired on a LSR II™ flow cytometer with three lasers (violet, blue, and red), whilst cells stained with other panels were acquired on a LSR II™ flow cytometer with four lasers (violet, blue, green and red). Acquired data was analysed on FlowJo™ software version 10 (FlowJo, LLC, Ashland, OR, USA).

Please see Appendix 8.1 for the list of antibodies and antibody panels used in this thesis.

2.5 Cell-sorting

2.5.1 Flow-assisted cell-sorting (FACS)

After staining PBMC and LILs with Antibody Panel 6 and undergoing RBC lysis as per above, cells were resuspended in 150μL PBS and kept at 4°C. Collection tubes (1.5mL microtubes, Eppendorf, supplied by Sigma-Aldrich, Gillingham, UK) were prepared one day prior by filling with sterile 10% BSA (diluted in D-PBS) and storing at 4°C overnight. Immediately prior to FACS, the 10% BSA was discarded, and the tubes were washed twice with D-PBS, then filled with 10μL D-PBS, so that cells would be sorted into only a very small volume and this would minimise subsequent centrifuge steps.

Cells were sorted on the BD FACS ARIA III, and gated on alive cells, singlets, CD45+ and lymphoid/myeloid population as shown in chapter 4.

2.5.2 Magnetic-assisted cell-sorting (MACS)

CD3+ T-cells were negatively selected via MACS from PBMC suspension using the Miltenyi Biotech Pan T-cell Isolation Kit as per the manufacturer's instructions. Briefly, up to 10^7 cells were stained with Pan T-cell Biotin-Antibody Cocktail and incubated at 4°C for 5 minutes. Subsequently, Pan T-cell Microbead Cocktail and buffer were added and incubated for a further 10 minutes at 4°C. This cocktail consisted of monoclonal antibodies against CD14, CD15, CD16, CD19, CD34, CD36, CD56, CD123, and CD235a (GlycophorinA).

An LS Column was then placed in the magnetic field of a MACS separator and prepared by rinsing with buffer. The stained cell suspension was then applied onto the column and the flow through was collected. This represented the enriched unlabelled T-cell population.

2.6 Cell Culture and ATRA stimulation

T75 culture flasks were prepared one day prior with a solution containing 3μg/ml anti-CD3 and 3μg/ml anti-CD-28 (4ml/flask, diluted in D-PBS) and incubated overnight at 4°C. The subsequent day, excess D-PBS was removed, and 10ml T-cell suspension (derived via MACS) at 1 x 10^6 cells/ml in complete growth media (RPMI-1640 with L-glutamine, 10% FCS, 1% penicillin/streptomycin), supplemented with 100U/mL recombinant human IL-2, was added into each of the T75 flasks. 100nM ATRA (diluted in 1:1000 DMSO) was added to flasks where cells were to undergo ATRA exposure. Cells were incubated in 37°C CO_2 incubators for 7-8 days. At 48-72 hours, fresh complete growth media, supplemented with IL-2 ±ATRA, was replaced. At day 7-8, cells were washed, spun down, and stained for flow cytometric analysis.

2.7 10X Genomics and Single cell RNAseq

2.7.1 Sample processing for 10x Genomics single-cell 3' library generation

Sorted myeloid and lymphoid populations from liver FNA samples and PBMC samples were prepared as detailed in the 10x Genomics Chromium Single Cell 3' v2 Reagent Kit user guide. Only samples which had a minimum of 2000 FACS-sorted cells were processed further for sequencing. Following sorting (for live, CD45+ myeloid/lymphoid lineage cells), cell viability and numbers were re-assessed via Trypan Blue and using a Neubauer haematocytometer prior to downstream processing. The cell stock concentration for

loading onto the 10x Genomics microfluidic chip was typically ~1,000 cells/μL, and a maximum of 10,000 cells were loaded per channel of the chip, thereby aiming for a cell multiplet rate of <5%. Following sample and reagent loading, the chip was placed into the 10x Genomics Chromium Single Cell Controller, which enables the cells to be partitioned into nanolitre-scale Gel Bead-In-Emulsions (GEMs), whereby the aim is to have a single cell and single 10x Barcoded Gel Bead enclosed in an oil droplet. As the cells are delivered at a limiting dilution, >90% of generated GEMs contain no cell, while the remainder largely contain a single cell.

The 10x GemCode Technology samples a pool of ~ 750,000 oligonucleotide barcodes to separately index each cell's mRNA. Following generation the GEMs were transferred to a twin.tec 96-Well PCR Plate and sealed with a Pierceable Foil Heat Seal (Bio-Rad) and a PX1 PCR Plate Sealer (Bio-Rad). Upon partitioning the Gel Bead dissolves and its oligonucleotide primers are released into the GEM's aqueous environment of the GEM; the cell captured in the GEM is also lysed. The released primers contain (i) an Illumina R1 sequence (read 1 sequencing primer), (ii) a 16 nt 10x Barcode, (iii) a 10 nt Unique Molecular Identifier (UMI), and (iv) a poly-dT primer sequence. The In-GEM reverse transcription into full-length, barcoded cDNA from the poly A-tailed mRNA transcripts was performed using a Veriti 96-well thermal cycler. The reverse transcription reaction is primed by the barcoded Gel Bead oligo and the reverse transcriptase incorporates the template switch oligo via a template switching reaction at the 5' end of the transcript. After incubation, the GEMs are "broken", pooling single-stranded, barcoded cDNA molecules from every cell, noting that cDNA molecules from any single GEM have all been labelled with the same 10x Barcode during the reverse transcription reaction. The pooled

fractions were then recovered using the 10x Genomics Recovery Agent and magnetic Dynabeads™ MyOne™ SILANE (Thermo Fisher) to remove the leftover biochemical reagents and primers from the post-GEM reaction mixture. The bulk cDNA was amplified by polymerase chain reaction (PCR) as per the manufacturers' guidelines, setting the total number of cDNA amplification cycles in relation to the estimated target cell recovery number so as to generate sufficient mass for library construction. SPRIselect Reagent was then used for post-cDNA amplification reaction clean-up, and samples were quality checked and quantified using the Agilent Bioanalyzer D5000 ScreenTape High Sensitivity chip (Agilent).

Enzymatic fragmentation, post-fragmentation end-repair, A-tailing, and double-sided size selection with SPRIselect Reagent (Beckman Coulter) were used to optimize the cDNA amplicon size prior to library construction. While R1 (read 1 primer sequence) was added to the molecules during GEM incubation, P5, P7, a sample index, and R2 (read 2 primer sequence) were added during library construction via end repair, A-tailing, adaptor ligation, and PCR. For the sample index PCR the total number of sample index cycles utilised was selected based on the amount of sample input used for the library construction, as per the manufacturers' protocol. The final libraries contained the P5 and P7 primers used in Illumina bridge amplification. Post-library construction quality checking was performed using the Agilent TapeStation High Sensitivity D1000 ScreenTape (Agilent). Library quantification was performed using the KAPA DNA Quantification Kit (KAPA Biosystems) for Illumina Platforms.

2.7.2 Single-cell library sequencing and quality checking

Sequencing of 10x Genomics Single Cell 3' libraries was performed by the Oxford Genomics Centre core sequencing facility (Wellcome Centre for Human Genetics, University of Oxford). Briefly, for sequencing libraries were pooled, denatured, and concentrations were adjusted according to the Illumina specifications, and sequenced on an Illumina HiSeq 4000, aiming for a sequence coverage of ~50,000 raw reads per cell. A Single Cell 3' library comprises standard Illumina paired-end constructs that begin and end with P5 and P7. The Single Cell 3' 16 bp 10x Barcode and 10 bp UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment. Sample index sequences are incorporated as the i7 index read. Read 1 and Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing. Thus, the single cell libraries were run using paired-end sequencing with single indexing, using 26 cycles for Read 1, 8 cycles for the i7 Index, and 98 cycles for Read 2.

Sequencing a Single Cell 3' Library produces a standard Illumina BCL data output folder. The BCL data include the paired-end Read 1 (containing the 16 bp 10x Barcode and 10 bp UMI) and Read 2 and the sample index in the i7 index read. For initial quality checking and data analysis, the 10x Genomics Cell Ranger analysis pipeline was used for alignment, gene counting, and data visualisation. Downstream data analysis was performed using the Seurat R package [176] and incorporating a custom normalization step to make samples comparable whereby the median nUMI for each sample was calculated and a scaling factor with the lowest number was created to enable downsampling. The Seurat R package was used for calculation of metrics generated from the raw data; data filtering; calculation of metrics generated from the raw data; estimation of cell and gene numbers;

principal component analysis; dimensionality reduction (using t-SNE); and marker expression analysis.

2.8 Immunohistochemistry (IHC)

5μm sections were cut from formalin-fixed, paraffin-embedded (FFPE) intestinal and liver biopsies and fixed onto charged microscope glass slides. Slides were warmed in an oven at 60°C for 1-12 hours to melt the paraffin. Slides then underwent two baths of Histo-clear and graded ethanol baths (2 x 100%, 1 x 75%, 1 x 50%) to clarify and de-paraffinise the sections and then placed in de-ionised water to rehydrate them.

Antigen retrieval was performed utilising an electric steamer, and incubating the cells in a bath of antigen retrieval buffer (10mM TRIS/1mM EDTA pH 9) for 20 minutes at minimum 80°C, then allowed to cool to room temperature. The slides were then placed in the ThermoShandon Sequenza™ system for blocking and staining of the sections.

Non-specific human FcR was blocked by incubating slides with 10% human serum (diluted in D-PBS) for 30 minutes at room temperature, followed by incubation with CD3 or CCR9 primary antibody. CCR9 antibody staining (1:500 for liver, 1:200 for intestine) was at 4°C overnight, whilst CD3 antibody staining (1:100) was at room temperature for 30 minutes.

Slides stained with anti-CCR9 underwent a second blocking step to prevent non-specific binding of goat FcR (as the biotinylation antibody was raised in goat) via incubation with 10% goat serum for 20 minutes at room temperature. Endogenous peroxidases were then blocked on all sections with 3% hydrogen peroxide for 15 minutes at room temperature.

For CCR9 antibody slides only, a biotinylation step was performed using biotinylated anti-rabbit antibody (1:67) for 30 minutes at room temperature. Subsequently, sections were incubated with VECTASTAIN® ABC reagent for 30 minutes as per the manufacturer's instructions. For CD3 antibody slides, biotinylation was not necessary to amplify the signal, and these slides were incubated with anti-mouse/rabbit secondary antibody (from the Dako enVision kit) for 30 minutes at room temperature.

Slides were then incubated with the substrate-chromogen solution as per the Dako enVision kit for 2-3 minutes and then counterstained with Mayer's haematoxylin for 20 seconds. Slides were then mounted with aqueous mounting solution and left to dry at room temperature.

Slides were digitally scanned using 40x objective equivalent using the Aperio Scanscope, and counting of positive cells per high power field (HPF, 5 in total) was carried out by pathologist, Dr Eve Fryer, using Aperio ImageScope and Image J Software.

2.9 Statistical analyses

For comparison of continuous variables between two groups with *normal distribution*, unpaired or paired T-test (as appropriate, two tailed) was used, and among three groups, Ordinary one-way ANOVA (unpaired data) or RM one-Way ANOVA (paired data) was used, with Tukey's multiple comparisons test.

For comparison of continuous variables between two groups with *non-normal distribution*, Wilcoxon matched-pairs signed rank test (paired data) or Mann Whitney test (unpaired data) was used, or, if among three groups, Kruskall-Wallis test (unpaired data) or Friedman test (paired data) was used with Dunn's multiple comparisons.

Comparisons of categorical outcomes and proportions was undertaken using 2x2 contingency tables with either Fisher's exact test or Chi-square test, depending on number of comparisons and number of outcomes involved.

All statistical analyses were carried out on GraphPad Prism version 8 for Mac OS (GraphPad Software, La Jolla California USA, www.graphpad.com) with the exception of univariate and multivariate analyses (R 3.5.1 Core Team (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2018. URL http://www.R-project.org/.) and RNAseq data analysis (described above).

3 Gut-Homing Lymphocytes in the Peripheral Blood and Intestine in PSC

3.1 Introduction

There is an intimate link between PSC and IBD, with the majority of patients with PSC having concomitant IBD. The IBD in PSC has its own unique phenotype as distinct from classical UC and CD, and there are limited data on differences between the conditions at an immunological level. There is a suggestion that gut-homing lymphocytes may travel from the inflamed intestine to the liver in PSC/IBD.

It is difficult and time-consuming to carry out translational research into lamina propria mononuclear cells (LPMC). This is due to the ethics and time involved in collecting human intestinal samples as well as the process of extracting the LPMC and carrying out various experiments, usually on freshly isolated cells. Add to this the rarity of PSC as a condition, it is not surprising that an evaluation of gut-homing lymphocytes within the gut in PSC/IBD has never been carried out.

Finally, there are limited data both within the peripheral blood and more so within the intestinal compartment with regards to the expression of gut-homing markers on T-cells in PSC/IBD, IBD and controls. In particular, there are conflicting data as to the accumulation of these cells in the inflamed versus uninflamed intestine in IBD and healthy controls.

3.2 Aims and Hypotheses

Given these uncertainties in the literature, I set out with the following aims:

1. *Determine the proportion of gut-homing lymphocytes* in the peripheral blood in patients with PSC/ as compared with healthy controls, liver controls, SD-PSC, and UC;*
2. *Determine the proportion of gut-homing lymphocytes* in the ileum and colon (both inflamed and uninflamed) in patients with PSC/IBD as compared with healthy controls and UC;*
3. *Analyse the relationship between gut-homing markers within the peripheral blood and intestine, as well as factors which may prime these markers.*

My chapter-specific hypotheses were as follows:

1. *There is a differing distribution throughout tissues (peripheral blood, intestine and liver) of gut-homing lymphocytes* in PSC/IBD versus controls.*
2. *There is a differing proportion of gut-homing lymphocytes* in inflamed colonic tissue compared with uninflamed colonic tissue.*

*Gut-homing lymphocytes as denoted by markers of CCR9, $\alpha 4\beta 7$ and CD161

3.3 Methods

Samples were obtained from patients under ethics of the Gastrointestinal Illnesses in Oxford Biobank study (REC Ref: 16/YH0247) and the Oxford IBD Cohort Study (REC Ref: 09/H1204/30). Peripheral blood was taken from patients with PSC (both with and without IBD), SD-PSC, UC, healthy controls (HC), and patients with other chronic liver

diseases (liver controls, LC). PBMC were isolated and stained for flow cytometric analysis with Panel 1 antibodies.

Intestinal biopsies were taken at flexible sigmoidoscopy or colonoscopy from patients with PSC/UC, UC and HC. Separate samples were taken from the ileum, inflamed colon and uninflamed colon where possible. LPMC were isolated and stained with Panel 1 antibodies for flow cytometric analysis. The proportion of various T-cell subsets which were positive for various gut-homing markers was compared among different cohorts.

Intestinal biopsies were taken in the same way at endoscopy and fixed in formalin and subsequently embedded in paraffin. 5μm sections were placed on microscopy slides, and IHC was performed for detection of CD3 and CCR9. Stained sections were scanned to create electronic images. The location of positive cells within the liver was described qualitatively. Five HPF areas (40X objective) with the highest density of CCR9 staining were determined by pathologist, Dr. Eve Fryer, and matching HPFs were captured on CD3 staining images. Only lymphocytes with discretely positive staining were counted. The mean counts of CCR9+ and CD3+ cells in 5 HPF were determined respectively and compared between PSC/UC and controls (UC and HC), as well as the CCR9/CD3 ratio.

3.4 Results

3.4.1 Peripheral blood mononuclear cell results

3.4.1.1 Baseline demographics

PBMC were available from 110 patients (PSC n=37, SD-PSC n=5, UC n=23, HC n=21, and LC n=24). Of those with PSC, 28/37 (75.7%) had IBD. Baseline demographics are shown in

Table 3.1. In the total population, 50 (45.5%) patients were male and the median age was 52.1 years (IQR 32.6-65.7, range 20.7-84.8). The only significant difference among populations was that LC patients were older than HC and UC patients, otherwise no differences in baseline demographics were found. The majority of LC patients had an autoimmune condition including PBC (66.7%, including two patients with overlap between PBC and autoimmune hepatitis (AIH)) and AIH (20.8%), with the remaining 5 patients (20.8%) having alcoholic liver disease (ALD) or non-alcoholic steatohepatitis (NASH).

3.4.1.2 Expression of gut-homing markers on PBMC

The median proportion of CD4+ T-cells which were CCR9+ was lower amongst patients with PSC (3.1% [IQR 2.5-4.0) compared with those with UC (4.0% [IQR 3.0-5.1], p=0.031), and was numerically lower compared with HC (3.8% [IQR 2.6-5.6], p=0.358, see *Figure 3-1* and *Figure 3-2*). There was no difference in the proportion of CD4+ T-cells which were CCR9+ when comparing among PSC/IBD patients, PSC/no IBD patients and SD-PSC patients. The median proportion of CD8+ T-cells which were CCR9+ among the PSC cohort was 2.5% (IQR 1.5-3.7) but there was no difference when compared with the various control cohorts.

Table 3.1 - Baseline demographics of patients from whom PBMC were isolated.

Variable	Healthy controls (n=21)	Ulcerative Colitis (n=23)	Liver controls (n=24)	PSC (n=37)	SD-PSC (n=5)	P-value
Male gender, n (%)	11 (52.4%)	8 (34.8%)	7 (29.2%)	22 (59.5%)	2 (40.0%)	0.106*
Age, median (IQR)	33.4y (28.2-45.1)	33.4y (28.2-45.1)	58.5y (52.4-70.1)	50.4y (28.0-64.5)	54.1 (33.6-70.7)	<0.0001#
IBD, n (%)	n/a	23 (100%)	n/a	28 (75.7%)	3 (60%)	-
Ulcerative Colitis	n/a	23 (100%)	n/a	26 (92.9%)	1 (33.3%)	-
Crohn's Disease	n/a	0 (0%)	n/a	2 (7.1%)	1 (33.3%)	-
IBD-unspecified	n/a	0 (0%)	n/a	0 (0%)	1 (33.3%)	-
Type of Liver Disease, n (%)						-
Primary Biliary Cholangitis	n/a	n/a	14 (58.3%)	n/a	n/a	-
Autoimmune Hepatitis	n/a	n/a	3 (12.5%)	n/a	n/a	-
PBC/AIH overlap	n/a	n/a	2 (8.3%)	n/a	n/a	-
Alcoholic liver disease	n/a	n/a	4 (16.7%)	n/a	n/a	-
Non-alcoholic steatohepatitis	n/a	n/a	1 (4.2%)	n/a	n/a	-

*Chi-square test applied. #Kruskall-Wallis test applied; Dunn's multiple comparisons test revealed differences were significant between HC vs. LC and UC vs. LC (p=0.0003 for both); all other comparisons were not statistically significant (including comparisons with PSC). AIH, autoimmune hepatitis; IBD, inflammatory bowel disease; n, number; n/a, not applicable; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SD, small duct.

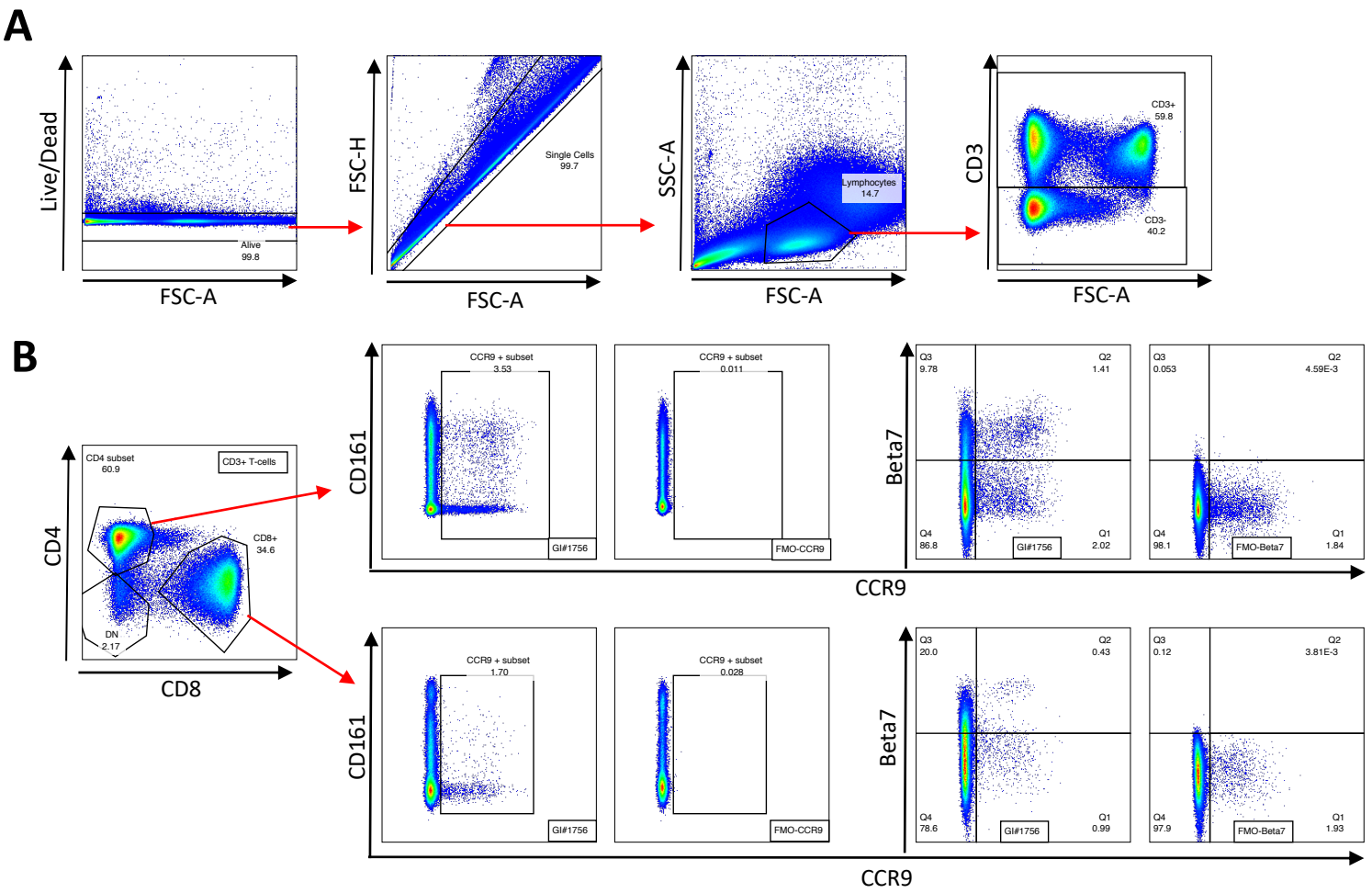


Figure 3-1 - Representative flow cytometry scatter plots and gating on PBMC of CD4+ and CD8+ T-cells and CCR9+ cells.

A, PBMC were gated on alive cells, single cells, lymphocytes and CD3+ T-cells. B, CD3+ T-cells were gated on CD4+ (upper panels) and CD8+ T-cells (lower panels). Cells were determined as CCR9+ according to an FMO sample and gating, as were cells which were double positive for β7 and CCR9 (quadrant Q2). Patient GI#1756 (LC: PBC) is shown as well as the relevant FMOs (as labelled). A, area; FMO, fluorescence minus one; FSC, forward scatter; H, height; LC, liver control; PBC, primary biliary cholangitis; PBMC, peripheral blood mononuclear cells; SSC, side scatter.

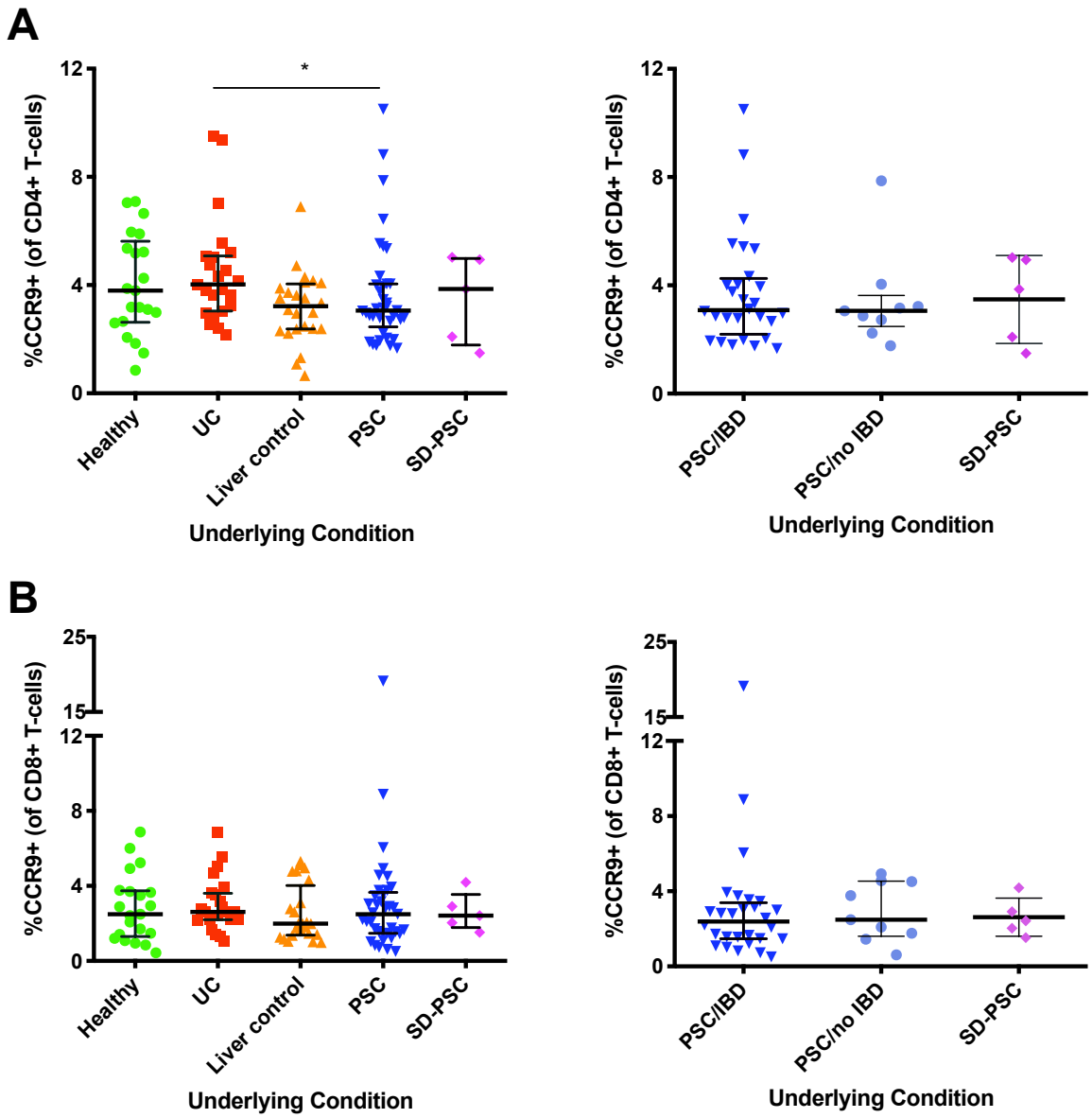


Figure 3-2 - The expression of CCR9 on CD4+ and CD8+ T-cells in the peripheral blood.

PBMC were isolated from patients with PSC (n=37), UC (n=21), LC (n=24), SD-PSC (n=5) and HC (n=20). Cell surface antibody staining and flow cytometric analysis was performed with gating on CD4+ (A) and CD8+ (B) T-cells. The proportion of T-cell populations which were CCR9+ was determined and compared between PSC and the various control groups (left-hand graphs). There was a reduced proportion of CD4+ T-cells which were CCR9+ in PSC compared with UC. No other differences were found. The right-hand graphs show the PSC cohort separated into those with IBD and those without IBD, and as compared with patients with SD-PSC. There was no difference in CCR9 expression on CD4+ and CD8+ T-cells among the three subgroups. Mann Whitney test was performed comparing PSC with control groups and median values and IQR are depicted. Unless stated, comparisons are not statistically significant ($p>0.05$). HC, healthy control; IBD, inflammatory bowel disease; LC, liver control; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; SD-PSC, small duct-PSC; UC, ulcerative colitis. *=$p<0.05$.

There was a lower median proportion of CD4+ T-cells which were β7+ numerically among patients with PSC (21.1% [IQR 16.5-28.3]) compared with HC (27.3% [IQR 18.8-33.0]) but this did not reach statistical significance (p=0.101, see *Figure 3-3* and *Figure 3-4A*). There was actually a lower median proportion of CD4+ T-cells which were β7+ among LC (17.7% [IQR 14.7-24.6]) compared with HC (p=0.020). There was no difference in the proportion of CD8+ T-cells which were β7+ between patients with PSC and controls.

When one looks at the cells which were double positive for both CCR9 and β7 (CCR9+/β7+), there was a lower median proportion among CD4+ T-cells in PSC (1.7% [IQR 1.2-2.3]) compared to HC (2.5% [IQR 1.7-3.7], p=0.006) and compared to UC (2.2% [IQR 1.5-2.6], p=0.039, see *Figure 3-1B* and *Figure 3-4B*). There was no difference in the proportion of CCR9+/β7+ cells among CD8+ T-cells among the various cohorts.

There was no difference among disease populations of the proportion of CD4+ T-cells which were CD161+ (see *Figure 3-4*). However, there were over three times as many MAITs (CD161++/Vα7.2+) in the CD8+ population in HC (median 3.8% [IQR 2.4-6.7]) as compared with the PSC cohort (1.2% [IQR 0.4-3.7], p=0.017). There were also numerically more CD8+ MAITs in the UC cohort (3.2% [IQR 0.9-7.3]) compared with PSC (p=0.095). Similarly, there was a low number of CD8+ MAITs in the LC cohort (1.0% [IQR 0.4-2.9], p=0.005 vs. HC). When analysing MAITs as a proportion of total CD3+ T-cells there were again less in PSC as compared with HC and UC (data not shown).

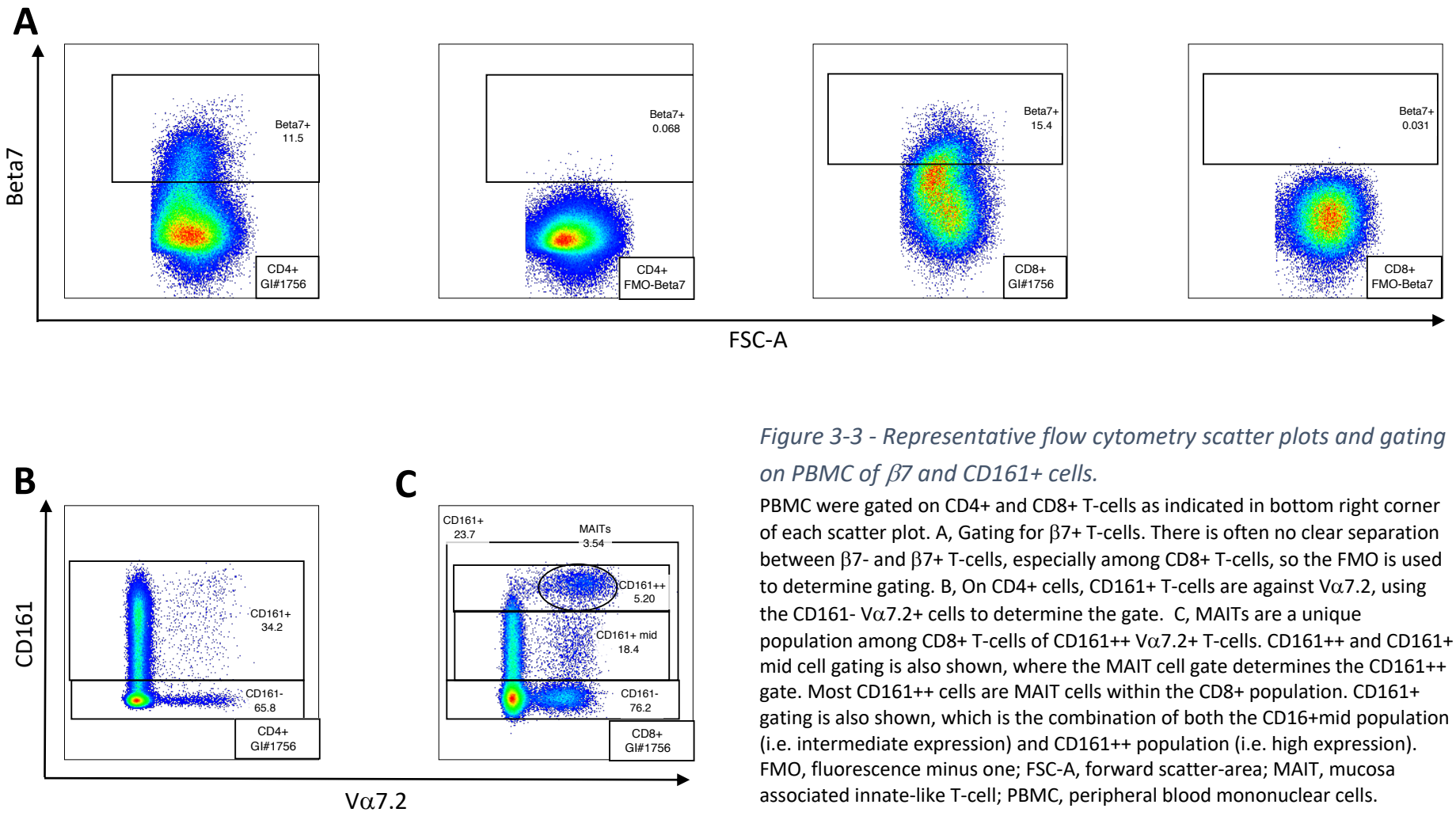


Figure 3-3 - Representative flow cytometry scatter plots and gating on PBMC of β7 and CD161+ cells.

PBMC were gated on CD4+ and CD8+ T-cells as indicated in bottom right corner of each scatter plot. A, Gating for β7+ T-cells. There is often no clear separation between β7- and β7+ T-cells, especially among CD8+ T-cells, so the FMO is used to determine gating. B, On CD4+ cells, CD161+ T-cells are against Vα7.2, using the CD161- Vα7.2+ cells to determine the gate. C, MAITs are a unique population among CD8+ T-cells of CD161++ Vα7.2+ T-cells. CD161++ and CD161+ mid cell gating is also shown, where the MAIT cell gate determines the CD161++ gate. Most CD161++ cells are MAIT cells within the CD8+ population. CD161+ gating is also shown, which is the combination of both the CD16+mid population (i.e. intermediate expression) and CD161++ population (i.e. high expression). FMO, fluorescence minus one; FSC-A, forward scatter-area; MAIT, mucosa associated innate-like T-cell; PBMC, peripheral blood mononuclear cells.

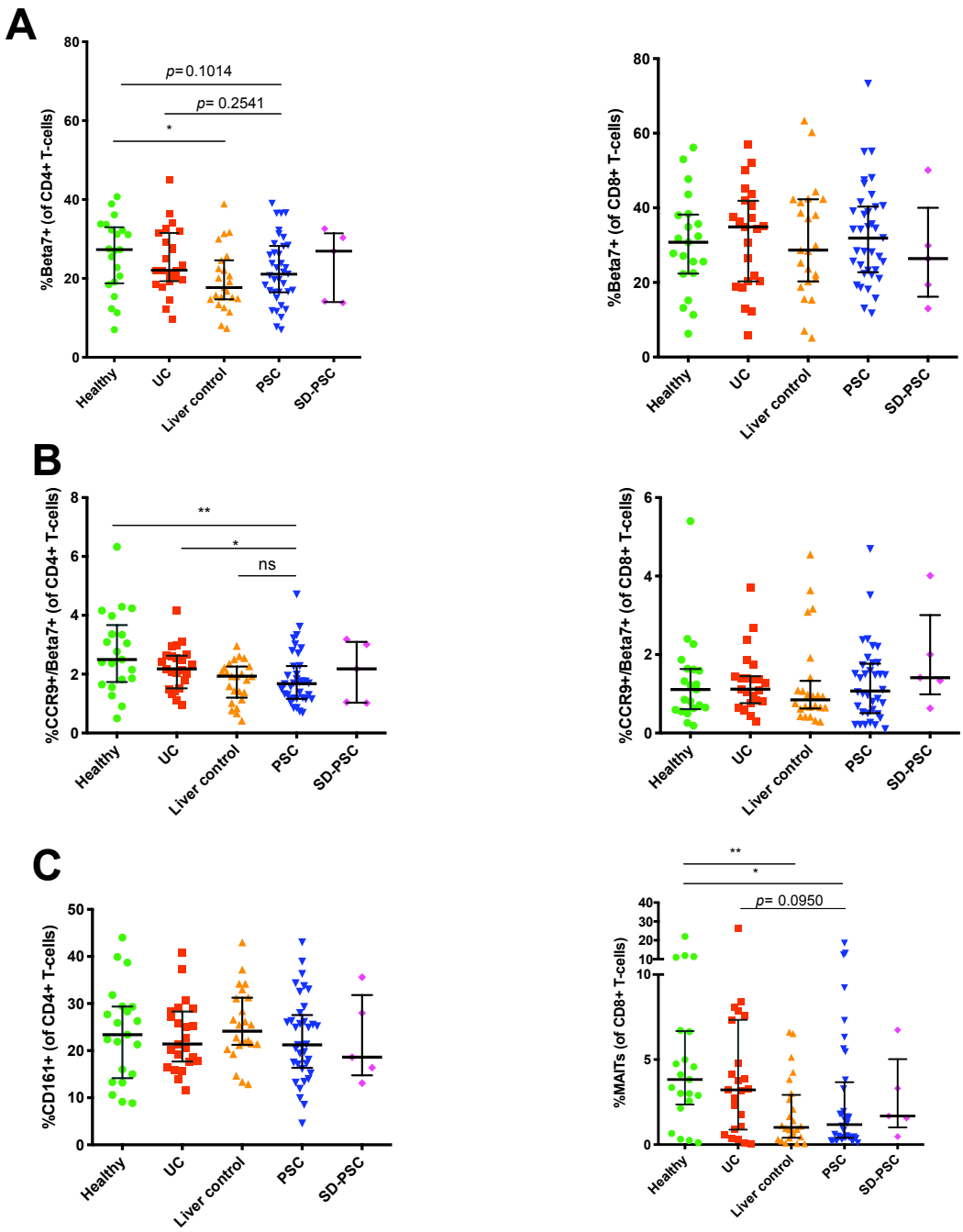


Figure 3-4 - The expression of β7 and CD161 on T-cells in the peripheral blood.

PBMC were isolated from patients with PSC and various other conditions and HC and stained with antibodies for flow cytometric analysis. Cells were gated on CD4+ T-cells (left-hand graphs) and CD8+ T-cells (right-hand graphs). A, there was a trend towards a reduced proportion of CD4+ which were β7+ among CD4+ T-cells in PSC vs. HC (left), but there was no difference in the proportion of CD8+ T-cells which were β7+ among different cohorts. B, There was a reduced proportion of CCR9+/β7+ CD4+ T-cells in patients with PSC compared to HC and UC (left) but there was no difference among CD8+ T-cells. C, Whilst there was no difference in the proportion of CD4+ T-cells which were CD161+ (left), there was a reduced proportion of MAITs among CD8+ T-cells in PSC compared to HC (right). All graphs show the median and IQR. Unpaired t-test and Mann Whitney tests were used between PSC and controls (and between LC and HC) depending on distribution of variables. Unless depicted, comparisons were not significant (p>0.05). HC, healthy control; LC, liver control; MAIT, mucosa associated innate-like T-cell; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; SD-PSC, small duct-PSC; UC, ulcerative colitis. *=p≤0.05; **p≤0.01.

3.4.1.3 Summary of peripheral blood results

There were some key findings within the peripheral blood, predominantly on CD4+ T-cells with regards to patients with PSC.

With regards to CD4+ T-cells, patients with PSC had:

- Slightly reduced proportions of CCR9+ T-cells
    - Statistically significantly less than UC
    - A trend towards less compared with HC
- A trend towards less β7+ T-cells compared with HC
- Less CCR9/β7 double positive T-cells
    - Compared with HC and UC
- No difference in CD161+ T-cells

With regards to CD8+ T-cells, the main findings were:

- Depressed MAITs in PSC
    - compared with HC
    - trend towards less compared with UC
- LC also had depressed MAITs.

The CCR9+/β7+ T-cells are the most gut-specific T-cells (as compared with positivity for either receptor/integrin alone). This depletion in these gut-homing CD4+ T-cells in PSC compared with UC (and in some cases HC) may indicate preferential homing of these T-cells out of the peripheral circulation and into tissue, whether it be intestine or elsewhere (such as liver). This may be brought about by increased expression of their ligands such as

CCL25 and MAdCAM-1, which has been shown to occur within the intestinal endothelium in IBD,[91] and within the liver sinusoidal endothelium in PSC.[137] Interrogation of the intestinal expression of these gut-homing T-cells is required to develop this theory further (further on in this chapter) as well as within the liver compartment (Chapter 4).

The findings of reduced CCR9+ CD4+ T-cells in PSC is slightly at odds with those of Papadakis *et al*. (raised CD4+ CCR9 in SB-CD compared with HC)[105] but they only found differences in patients with small bowel disease, not colonic, and furthermore did not examine patients with PSC. Meanwhile, these data are in keeping with those data from Gwela *et al.* suggesting reduced CCR9 and β7 CD4+ T-cells in PSC compared with HC, though they found reduced levels also in UC alone, which is at odds with my data.[84] They however examined CD4+ memory T-cells rather than all CD4+ T-cells as I did, and therefore direct comparisons cannot be made.

The findings here of reduced MAITs within the peripheral circulation in PSC and LC as compared with HC are in keeping with reduced MAITs previously seen in the literature in IBD and chronic liver disease.[177, 129-132] This relative reduction may be due to increased apoptosis of MAITs or potentially due to homing to tissue locations.

3.4.2 Lamina propria mononuclear cell results

3.4.2.1 Baseline demographics

82 LPMC samples were available from patients with PSC, UC, and HC. Samples consisted of ileal, inflamed colon and/or uninflamed colon biopsy samples from 51 unique patients (PSC n=17, UC n=15, HC n=19). The mean age was greater for HC (60.6y ±SEM3.9)

compared with PSC (39.9y ±4.0, p=0.0019) and UC (39.9y ±4.6, p=0.0027). There was no difference in the proportion of males in each group (PSC 64.7%, UC 40.0%, HC 57.9%, p=0.355). Of the 17 patients with PSC, 16/17 had concomitant IBD (UC n=15, CD n=1), and one patient had no IBD, but only an uninflamed colonic sample was taken from this patient. None of the patients with IBD were on a biologic at the time of sampling.

3.4.2.2 Predominance of gut-homing lymphocytes according to intestinal location and presence of inflammation

I examined the proportion of CD4+ T-cells which were positive for gut-homing markers CCR9, β7, and CD161 and compared these proportions among ileum, uninflamed colon and inflamed colon (see *Figure 3-5*).

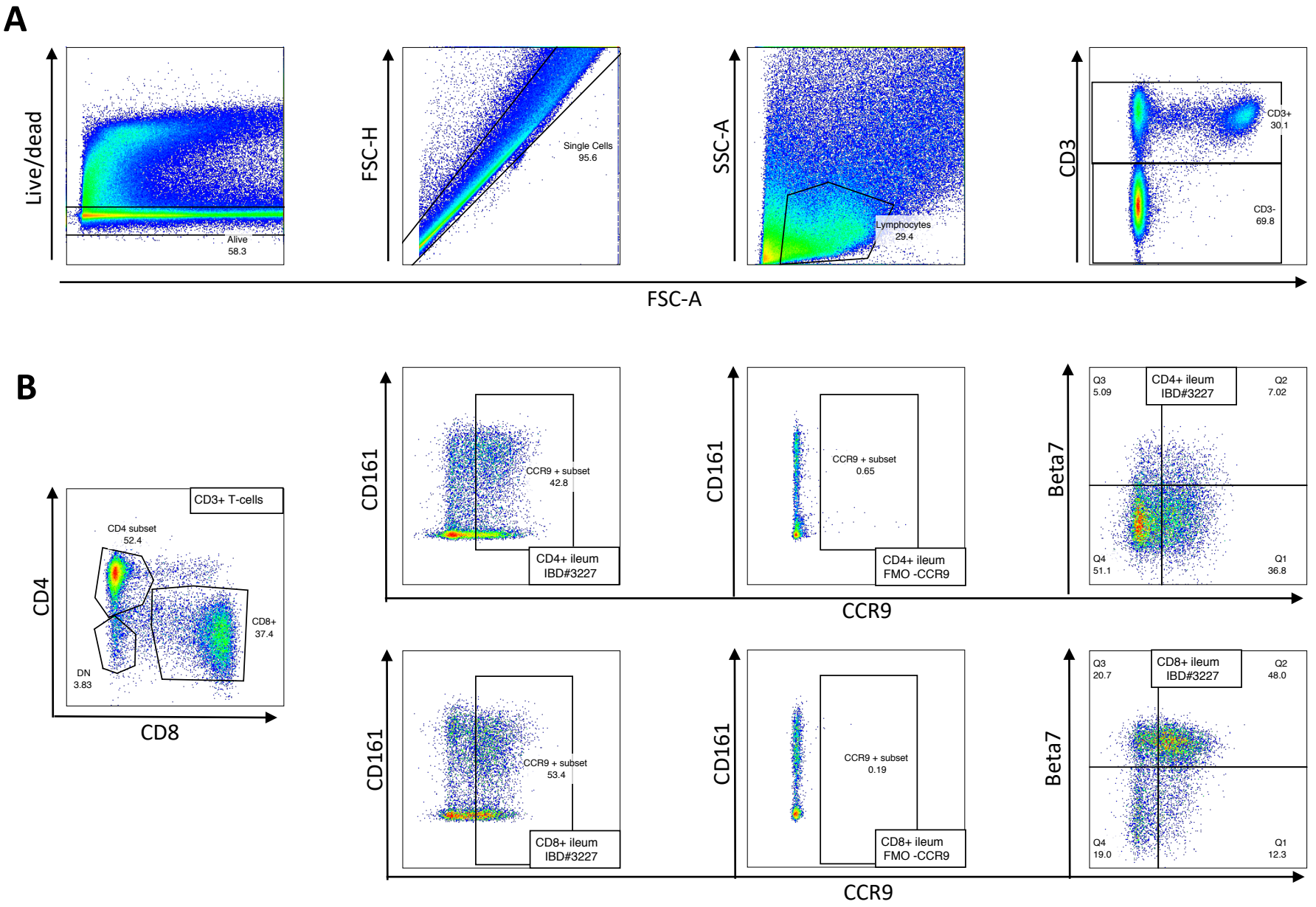
In keeping with the current literature, I found that there was a higher proportion of CCR9+, β7+ and CCR9+/β7+ T-cells (both CD4+ and CD8+) within the ileum as compared with the colon (both when comparing to uninflamed colon and inflamed colon in most instances, see Figure 3-6). This was particularly the case for CCR9 expression (and dual CCR9/β7 expression) where there was a wide separation between ileal T-cells and uninflamed colonic T-cells. Furthermore, there was significant enrichment of CCR9+ T-cells within the tissue compartment (ileum/colon) as compared with PBMC, confirming their gut-homing phenotype. There was no enrichment (or depletion) of CCR9+ T-cells within the inflamed colon as compared with the uninflamed colon.

Interestingly, there was a lower median proportion of β7+ cells in the inflamed colon compared with the uninflamed colon, both among CD4+ T-cells (13.1% vs. 15.9%

respectively, p=0.019) and particularly among CD8+ T-cells (39.0% vs. 60.3%). These analyses were unpaired, however, when analysing these comparisons among the 9 patients with paired inflamed and uninflamed colonic biopsies, the differences remained the same (both numerically, and statistically significant, data not shown). CD4+ β7+ T-cells did not appear to be enriched within the ileum/colon as compared with peripheral blood, however, CD8+ β7+ T-cells were enriched within the intestine, particularly within the ileum.

With regards to CD161 expression, there was no difference between ileum and colonic samples with regards to the proportion of CD4+ T-cells which were CD161+. Likewise, there was no predominance of MAITs among CD8+ T-cells within one of these tissue compartments. What was more significant, was that the median proportion of CD4+ T-cells which were CD161+ was much higher in the ileum (50.3%), uninflamed colon (58.1%) and inflamed colon (49.9%) as compared with in the peripheral blood (22.3%, $p<0.001$ for all comparisons of PBMC vs. tissue).

This was not the case for MAIT cells among the CD8+ T-cell population where the median proportions within tissue (ileum 1.3%, uninflamed colon 0.8%, inflamed colon 1.1%) were similar to those within peripheral blood among the various cohorts of patients described in 3.4.1.2 (1.8%, comparisons all non-significant except for PBMC vs. uninflamed colon, p=0.017). In any case, there is no enrichment (nor a depletion) of CD161+ CD4+ T-cells or MAIT cells within the inflamed colon compared with the healthy/ uninflamed colon.


A
Live/dead
Alive
58.3
FSC-H
Single Cells
95.6
SSC-A
Lymphocytes
29.4
CD3
CD3+
30.1
CD3-
69.8
FSC-A
B
CD3+ T-cells
CD4 subset
52.4
CD8+
37.4
DN
3.83
CD4
CD8
CD161
CCR9 + subset
42.8
CD4+ ileum
IBD#3227
CCR9 + subset
0.65
CD4+ ileum
FMO -CCR9
CCR9
Beta7
Q3
5.09
CD4+ ileum
IBD#3227
Q2
7.02
Q4
51.1
Q1
36.8
CCR9 + subset
53.4
CD8+ ileum
IBD#3227
CCR9 + subset
0.19
CD8+ ileum
FMO -CCR9
Q3
20.7
CD8+ ileum
IBD#3227
Q2
48.0
Q4
19.0
Q1
12.3

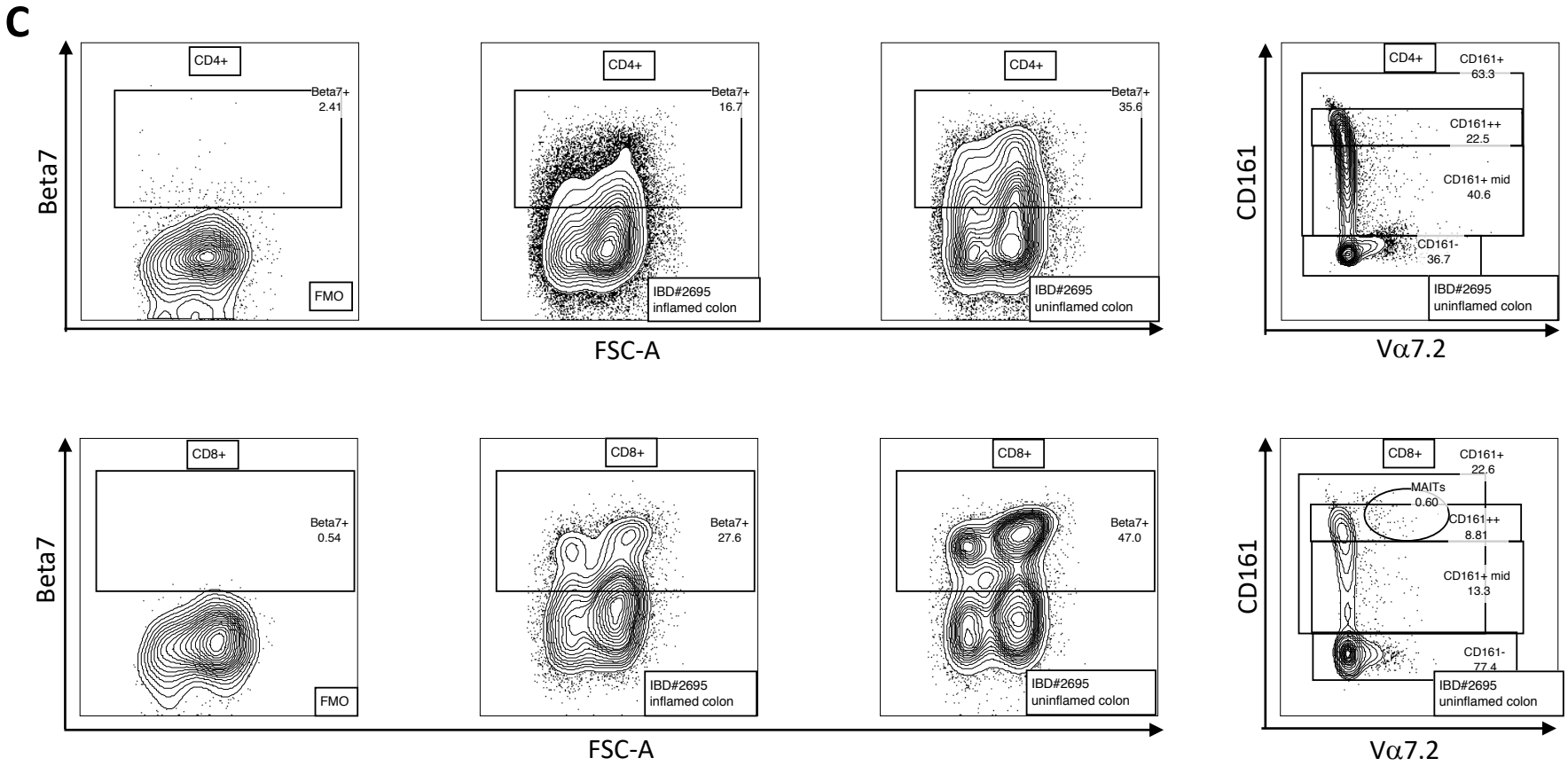


Figure 3-5 - Representative flow cytometry plots of ileal and colonic LPMC

LPMC were isolated from ileal, uninflamed colonic and inflamed colonic biopsies and underwent flow cytometric analysis. A, Cells were gated on alive cells, singlets, lymphocytes and then CD3+ T-cells. B. CD3+ T-cells were gated on CD4+ and CD8+ T-cells and then evaluated for the proportion of CD4+ T-cells (upper panels) and CD8+ T-cells (lower panels) which were CCR9+ according to a FMO, as well as evaluated for CCR9/β7 double positive cells (upper right quadrant, Q2). C, CD4+ T-cells (upper panels) and CD8+ T-cells (lower panels) were evaluated for the proportion which were β7+ (according to a FMO) as well as the proportion CD161+ and CD161++/Vα7.2+ (MAITs). A, area; FMO, fluorescence minus one; FSC, forward scatter; H, height; LPMC, lamina propria mononuclear cells; MAIT, mucosa associated innate-like T-cells; SSC, side scatter.

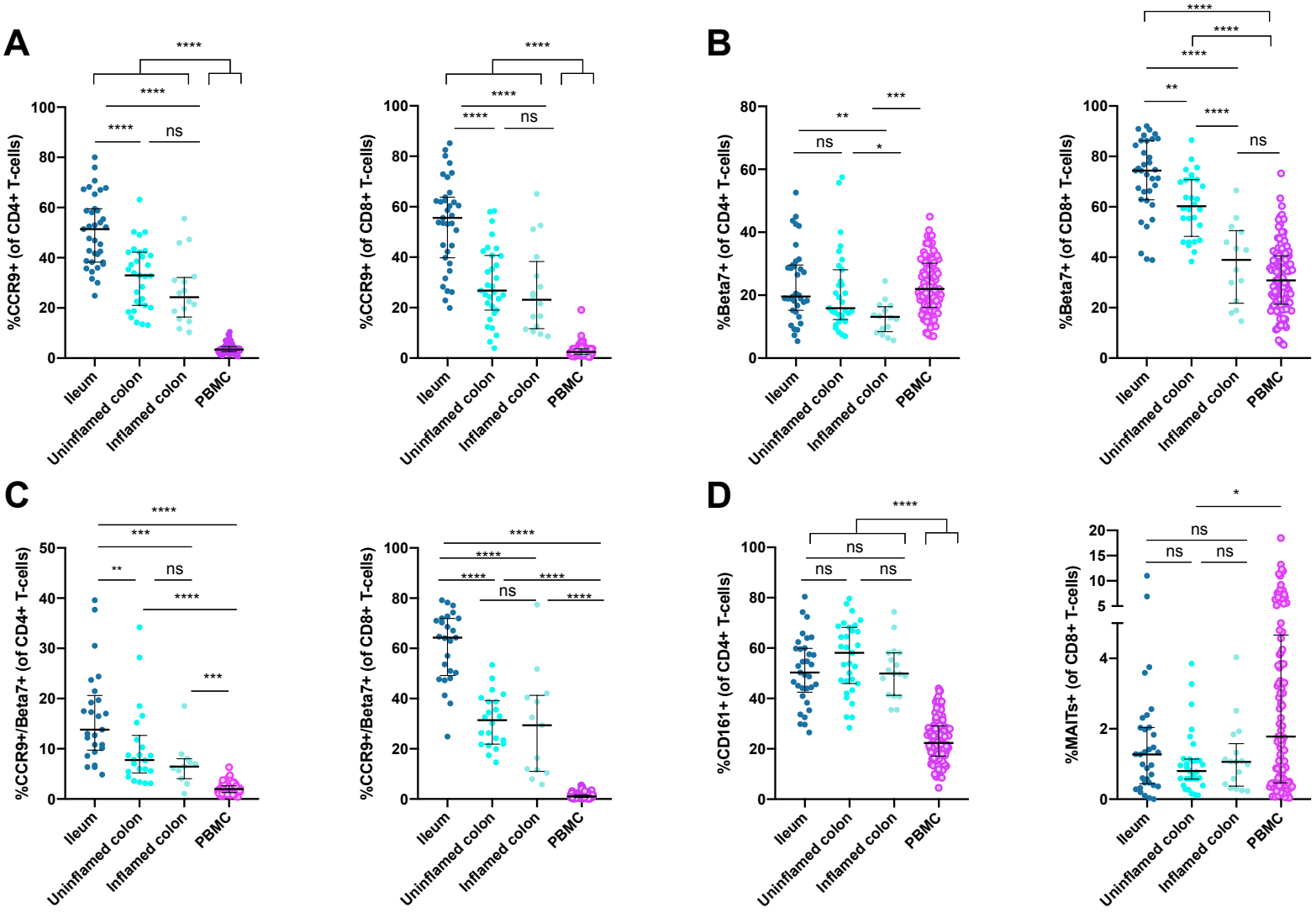


Figure 3-6 - Proportion of gut-homing T-cells among different tissue compartments as measured by flow cytometry.

Figure 3-6 - Abundance of gut-homing T-cells among different tissue compartments as measured by flow cytometry

LPMC were derived from biopsies taken at endoscopy from the ileum (n=35), uninflamed colon (n=31) and inflamed colon (n=16). Patients had PSC, UC or were HC. PBMC were isolated from patients with the same conditions (n=110). The proportion of CD4+ (left-hand graphs in each panel) and CD8+ graphs (right-hand graphs in each panel) which were positive for various gut-homing markers was compared among tissue compartments. A, The proportion of CCR9+ cells among CD4+ and CD8+ populations was highest in the ileum, followed by uninflamed and colonic inflamed colon, with relatively few in the peripheral blood. B, Ileal and colonic concentration of β7+ T-cells was only apparent among CD8+ T-cells and not CD4+ T-cells. There was a lower proportion of CD4+ and CD8+ T-cells which were β7+ in inflamed colon compared with uninflamed colon. C, CCR9/β7 double positive cells were particularly concentrated in the intestinal tissue compared with PBMC. D, There is an enrichment in the ileum and colon for CD4+ CD161+ T-cells as compared with the blood, but this is not the case for MAIT cells among the CD8+ T-cell population. All graphs show the median ±IQR. Unpaired t-test or Mann Whitney test performed for each comparison, depending on distribution of variables. HC, healthy controls; LPMC, lamina propria mononuclear cells; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; UC, ulcerative colitis. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

3.4.2.3 Comparison between PSC and controls in abundance of intestinal gut-homing T-cells

I compared the proportions of CD4+ and CD8+ T-cells which were positive for the various gut-homing markers between PSC and controls (UC and HC) within each of the three tissue compartments (ileum, uninflamed colon, and inflamed colon, see *Figure 3-7*).

The principal difference found was within the ileum. Patients with PSC had a lower mean proportion of CD4+ T-cells which were CCR9+ (42.9% ±SEM 2.8) compared with UC (57.0% ±3.6, p=0.005) and HC (55.7% ±5.1, p=0.026). This was also true among the CD8+ T-cell population for PSC vs. UC (45.9% ±4.3 vs. 61.3% 4.4, p=0.023), and numerically so for PSC vs. HC, but not to statistical significance (45.9% ±4.3 vs. 57.5% ±6.2, p=0.127). There was no difference in CCR9 expression on CD4+ and CD8+ T-cells in both the inflamed colon and uninflamed colon between PSC and controls.

There was no difference in the abundance of β7+ T-cells, CCR9+/β7+ T-cells (data not shown), CD4+ CD161+ T-cells and CD8+ MAIT cells in the various tissue compartments between PSC and controls.

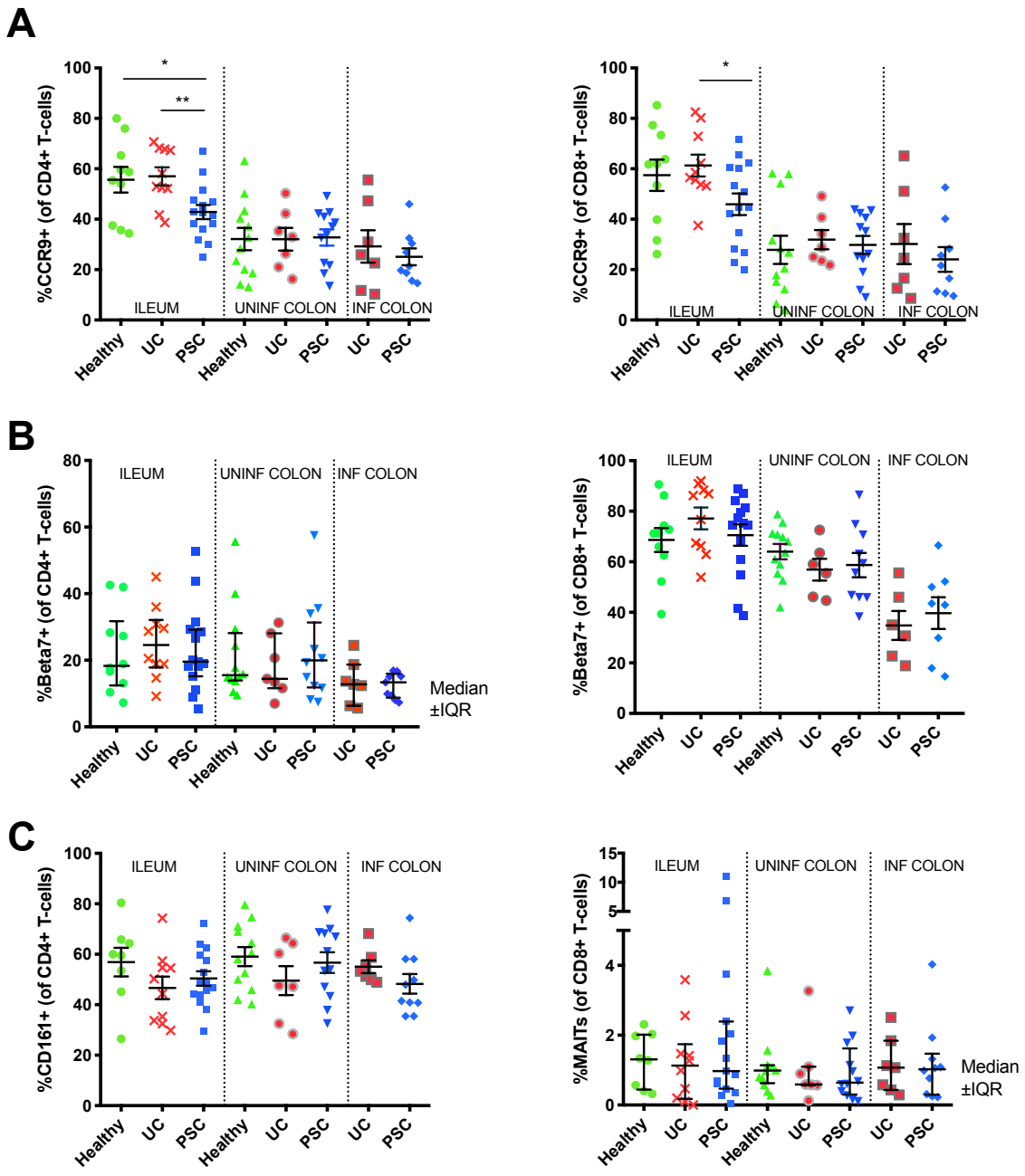


Figure 3-7 - Flow cytometric analysis of the abundance of gut-homing T-cells in the various intestinal tissue compartments between patients with PSC and controls

LPMC were isolated from the ileum, uninflamed colon and inflamed colon from patients with PSC (n=17), UC (n=15) and HC (n=19). Cells were stained with antibody Panel 1, then analysed by flow cytometry and gated on either CD4+ T-cells (left-hand graphs) or CD8+ T-cells (right-hand graphs). A, there was a lower proportion of CD4+ and CD8+ T-cells which expressed CCR9 in the PSC ileum compared with UC ileum and HC ileum, but there were no differences in the inflamed and uninflamed colon. B, within each tissue compartment, there were no differences between PSC and controls with regards to β7 expression on CD4+ and CD8+ T-cells. C, There was no differences between PSC and controls in the proportion of CD4+ T-cells which were CD161+ (left) nor the proportion of CD8+ T-cells which were MAITs (CD161++ Vα7.2+, right). Comparisons made between PSC and each control group individually in each tissue compartment with unpaired t-test or Mann Whitney test depending on distribution of values. Statistically significant results indicated. All graphs show mean ±SEM unless specified. HC, healthy controls; inf, inflamed; LPMC, lamina propria mononuclear cells; MAIT, mucosa-associated innate-like T-cells, PSC, primary sclerosing cholangitis; UC, ulcerative colitis; uninf, uninflamed.
*p=0.01-0.05; **p=0.001-0.01.

3.4.2.4 Summary of intestinal results and interim discussion

In this section, I have shown that:

1. There is an abundance of specific cell subsets within the gut compared with the blood:
    - CCR9+ T-cells - ileum > colon > PBMC
    - CCR9+/β7+ T-cells – ileum > colon > PBMC
    - CD8+ β7+ T-cells (not CD4+) – ileum > colon > PBMC
    - CD4+ CD161+ T-cells – ileum/colon > PBMC

2. There is differing β7 expression (but not CCR9) between inflamed and uninflamed colon:
    - Lower proportion of β7+ T-cells (both CD4+ and CD8+) in inflamed versus uninflamed colon

3. There were less ileal CCR9+ T-cells in PSC compared with UC and HC

The first finding is entirely in keeping with the literature for CCR9+ T-cells, though mapping of β7+ T-cells within the gut has not been carried out as extensively as for CCR9. It is interesting that CD4+ β7 T-cells do not preferentially occupy ileum versus colon, given that vedolizumab (α4β7 antagonist) works just as well for colonic IBD (if not better) than small bowel IBD.[178, 179] It also confirms that the antibody being used is specific for the gut-homing marker CCR9, which is important for when I utilised the same CCR9 antibody for experiments analysing liver-infiltrating lymphocytes (LILs) in the next chapter.

The finding of enrichment in the gut for CD4+ CD161+ T-cells has been seen in a small cohort of patients with CD and HC previously[180] and is in keeping with unpublished findings by another member in my group who analysed healthy and inflamed small bowel and colonic resections (Leng T, unpublished). Dr Leng also showed these cells have similar properties to MAIT cells, in that they are predominantly an effector memory phenotype and respond to both innate and adaptive signals. They were not enriched in inflamed colon compared with uninflamed colon, and further research on their role within the gut, particularly within inflammatory bowel disease, is warranted.

The reduced expression of β7 on T-cells in inflamed compared to non-inflamed colon is perplexing and at first counterintuitive. Given that one presumes $\alpha 4\beta 7$+ T-cells are recruited to the bowel in the setting of chronic inflammation (and therefore IBD), one would expect a higher amount of these cells to be present. It has been shown that in the setting of chronic inflammation (IBD) there is recruitment also of naïve T-cells to the ileum and colon which does not occur in health.[181] It is possible that the lower proportion of β7+ T-cells in inflamed colon is due to dilution from the number of naïve (and potentially non-β7+) T-cells. Another possibility is that $\alpha 4\beta 7$ is downregulated/internalised on entering the lamina propria – this has not been studied before.

Finally, the finding of altered proportions of CCR9+ T-cells within the ileum in PSC versus controls is an intriguing and important observation. This is particularly relevant for two reasons. Firstly, CCR9+ T-cells are concentrated in the ileum (not the colon). Secondly, PSC/IBD is associated with a significantly increased involvement of the ileum (backwash

ileitis) compared with UC alone.[3] In this cohort of 25 ileal samples from IBD patients (PSC n=15, UC n=10), histological examination of the ileum was available in 21. Histologic inflammation was present in 8/13 PSC ileal samples (61.5%) versus 3/9 UC ileal samples (33.3%, p=0.193). Papadakis *et al.* showed reduced proportions of CCR9+ T-cells in inflamed ileum compared with adjacent uninflamed ileum. The reduced ileal CCR9 expression in PSC may represent chronic inflammation (and potential resultant internalisation of the CCR9 receptor). Another possibility is that these CCR9+ T-cells are leaving the ileal lamina propria and re-entering the circulation. Hypothetically, this could be via the portal circulation to the liver and occurring more frequently in PSC/IBD compared with UC alone. The presence of these cells within the PSC liver will be explored further in Chapter 4.

3.4.3 Association of CCR9 and β7 with CD161 expression on T-cells

The abundance of CD4+ CD161+ T-cells within the ileum and colon led me to investigate whether there was an association of CD161 with CCR9 or $\alpha 4\beta 7$. I analysed flow cytometry data from PBMC and LPMC described earlier in this chapter. CD4+ T-cells and CD8+ T-cells were gated on CD161-, CD161+mid and CD161++ gates (the gates within the CD4 T-cells were derived from the CD8+ gates) and the proportion which were CCR9+ or $\beta 7$+ within these three CD161 populations were compared.

In PBMC, there was a striking increase in the proportion of CD4+ T-cells which were CCR9+ with increasing CD161 intensity (see *Figure 3-8*). This was also true in the tissue, whether examining individually within each tissue compartment (ileum, uninflamed colon, data not shown) or combining them. However, the difference from CD161- to CD161++ within the tissue was not quite so proportionately different as within PBMC.

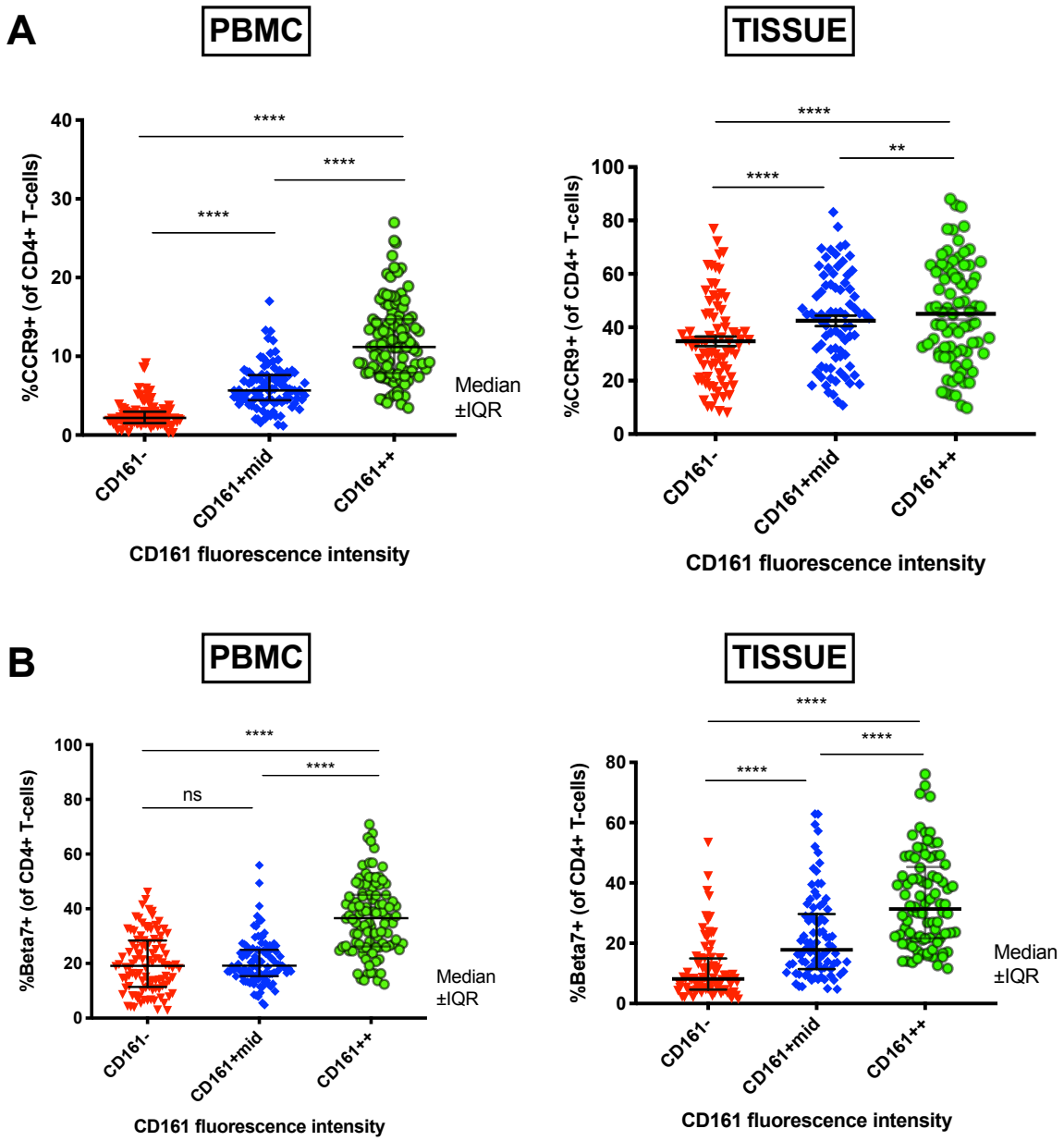


Figure 3-8 - The expression of CCR9 and β7 on CD4+ T-cells according to CD161 expression

PBMC were isolated from patients with PSC, SD-PSC, UC, LC and HC. LPMC were isolated from ileal, uninflamed colonic, and inflamed colonic biopsies from patients with PSC, UC and HC. PBMC and LPMC underwent flow cytometric analysis, cells were gated on CD4+ T-cells and the proportion of cells which were CCR9+ and β7+ was determined. A, There were increasing CD4+ T-cells which were CCR9+ with rising CD161 expression among PBMC (left) and to a lesser extent on LPMC (right). B, Similarly, there were increasing proportions of CD4+ T-cells which were β7+ with rising CD161+ expression (particularly high CD161 expression [CD161++]) among PBMC (left) and LPMC (right). Mean ±SEM is depicted unless otherwise stated. One-way ANOVA with Tukey's multiple comparison test or Friedman test ANOVA with Dunn's multiple comparison test was performed depending on distribution of data. HC, healthy controls; LC, liver controls; LPMC, lamina propria mononuclear cells; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; SD-PSC, small duct-PSC; UC, ulcerative colitis.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

The differing expression of CCR9 and β7 on CD8+ T-cells according to CD161 expression in peripheral blood and lamina propria was not clear cut and differed between PBMC and LPMC (data not shown). Significant compensation had to be applied to the fluorochromes on CD161 (PE) and β7 (PerCP-Cy5.5), so, given the conflicting data on these subsets, it was felt that comparisons of β7 among the CD161 subgroups from these data could not be confidently relied upon.

3.4.4 ATRA stimulation and the effect on T-cell expression of CCR9, β7 and CD161

It is known that T-cells are primed to become CCR9 and α4β7+ within GALT in the presence of ATRA.[98] Given the association of CCR9 and β7 with CD161, it was hypothesised that this interaction may potentially also prime T-cells to become CD161+. Therefore, we set out to test this hypothesis by stimulating T-cells *in vitro* in the presence of ATRA.

Magnetically sorted peripheral blood T-cells from 3 healthy donors (over the course of two experiments) were stimulated with anti-CD3 and anti-CD28 antibodies and cultured with IL-2 in the presence or absence of ATRA for 7-8 days. They were then stained with antibody Panel 4 and underwent flow cytometry analysis. The proportion of CD4+ and CD8+ memory T-cells (CD45RA-) which were positive for CCR9, β7, or CD161 were compared between those exposed to ATRA, and those exposed to vehicle.

Consistent with the literature the mean proportion of CD4+ memory T-cells which were positive for CCR9+ rose significantly in the presence of ATRA compared with no ATRA (3.1% ±SEM 1.3 vs. 26.3% ±5.4, p=0.010, see *Figure 3-9)*. This was numerically so for CD8+

memory T-cells but not to statistical significance (29.6% ±14.7 vs. 61.1% ±2.8, p=0.151). This was also true for the proportion of CD4+ T-cells (but not CD8+) which were β7+.

With regards to changes in CD161 expression in the presence of ATRA, there was a numerical rise in the median proportion of CD4+ memory T-cells which were CD161+ but not to statistical significance (8.2% [IQR 4.2-8.3] vs. 14.1% [IQR 5.6-14.3], p=0.250). There was no difference in the proportion of CD8+ CD45RA- T-cells which were CD161+ with regards to ATRA exposure.

These data do not show that ATRA causes CD161 expression as it does CCR9 and β7, though this may be potentially due to underpowering due to the small samples size, particularly among CD4+ memory T-cells given the numerical but non-significant rise in this subset. Nevertheless, the numerical rise was small (median 8.2% to 14.1%) compared with the significant rise of CCR9+ T-cells (3.1% to 26.3%) so it is unlikely that even with larger numbers of samples, that a true significant association between ATRA exposure and CD161 induction would be drawn.

Another group analysed this and also found a higher expression of CCR9 on CD4+ CD161+ T-cells as compared with CD161- T-cells.[182] They stimulated and cultured CD4+ T-cells (though for only 48 hours) with and without ATRA (n=6) and similarly found no significant increase in CD161 expression with ATRA. Therefore, it is unlikely that the association between CD161 and CCR9/β7 expression is due to ATRA upregulating CD161. It is possible that CD4+ CD161+ T-cells are enriched in the gut due to this inherent association with CCR9 and β7 expression, but the reason for their association is unclear.

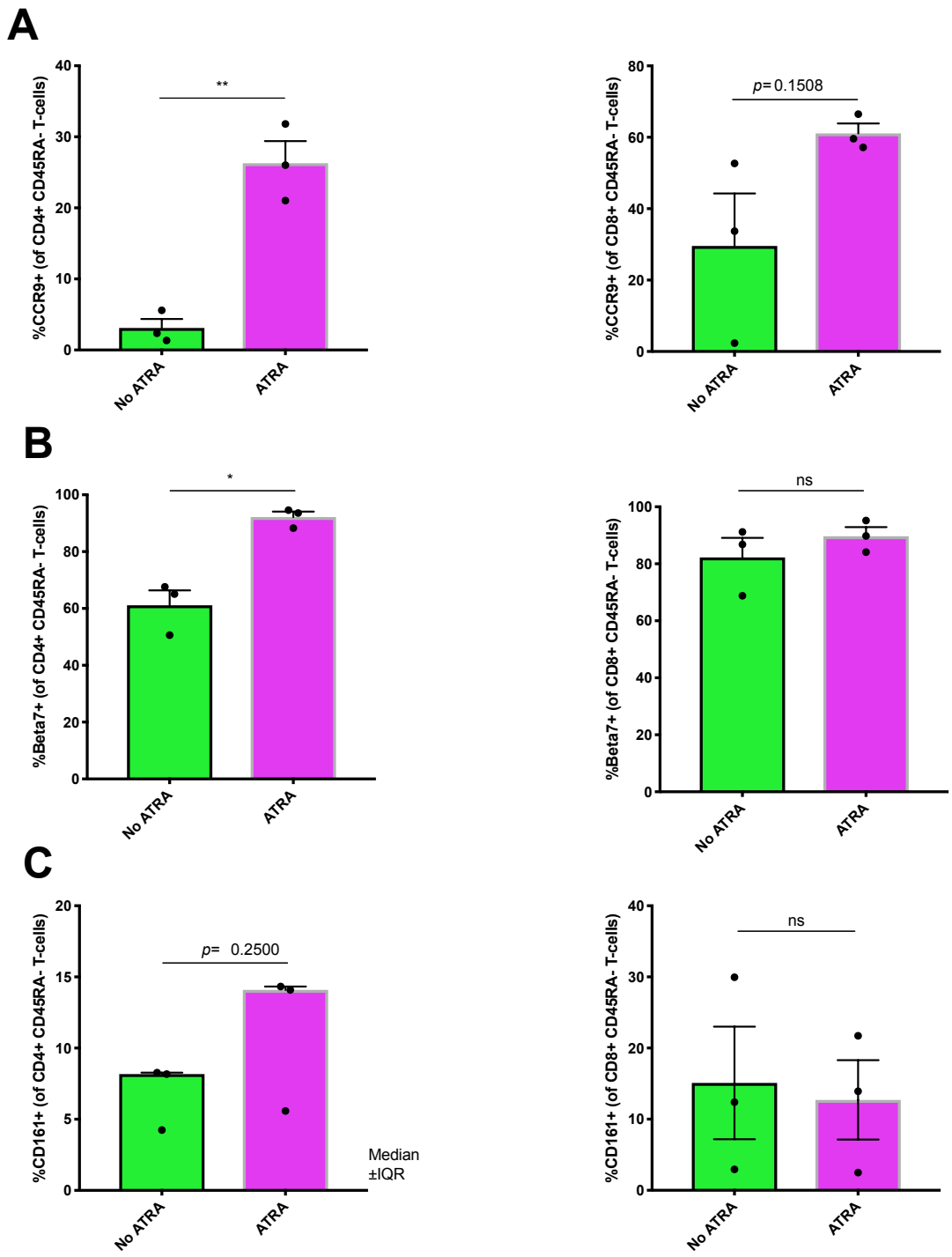


Figure 3-9 - Expression of various gut-homing markers on T-cells after exposure to ATRA.

Peripheral blood T-cells underwent TCR stimulation (anti-CD3 and anti-CD-28) in the presence or absence of ATRA, then underwent flow cytometric analysis. The proportion of CD4+ T-cells (left-hand graphs) and CD8+ T-cells (right-hand graphs) which were positive for CCR9, β7 and CD161 were compared between those which were exposed to ATRA and those which were not. A, The proportion of CD+ and CD8+ T-cells which were CCR9+ significantly increased with ATRA exposure. B, The proportion of CD4+ T-cells (but not CD8+ T-cells) which were β7+ increased significantly with ATRA exposure. C, there was no difference in the proportion of CD4+ and CD8+ T-cells which were CD161+ with ATRA exposure. Graphs show mean ±SEM unless reported otherwise. Paired t-test or Wilcoxon matched-pairs signed rank test was performed depending on distribution of variables. ATRA, all-*trans*-retinoic acid; TCR, T-cell receptor. *p=0.01-0.05; **p=0.001-0.01; ns=not significant, i.e. p>0.05.

3.4.5 Analysis of intestinal expression of CCR9 in PSC and controls with immunohistochemistry

Given the finding of reduced CCR9+ T-cells within the ileum in PSC versus controls, I sought to validate this finding by identifying and quantifying the CCR9 protein *in situ* in PSC intestinal specimens versus control specimens.

There is limited to no data on CCR9 expression in tissues spatially. The most obvious experimental way to analyse this is IHC, but antibodies which detect CCR9 reliably in tissue specimens, particularly FFPE specimens, are lacking. Previous attempts in our lab have failed to reliably identify CCR9+ lymphocytes in FFPE intestinal specimens due to significant background staining due to epithelial staining by the antibody.

I tested 5 different anti-human CCR9 antibodies, distinct from those already tested in our lab, and identified an antibody which stained via IHC lymphocytes within the intestinal lamina propria within FFPE specimens with minimal to no background staining (Abcam ab140791). I optimised the staining protocol by identifying the best antibody concentration and retrieval buffer, using various blocking steps to reduce background staining, and biotinylating the primary antibody and utilising the ABC system to strengthen positive staining. The resultant IHC protocol is described in *Section 2.8*.

I obtained FFPE specimens (ileal and colonic) from 15 patients with PSC/UC, and relevant controls (UC n=17, HC n=14) . I stained these specimens via IHC for CD3 and CCR9 and had the slides scanned in electronically using the Aperio digital pathology slide scanner. Images were viewed on Image J and the 5 high power fields (HPF) which had the highest CCR9 counts were identified, both within the ileum and colon. The corresponding 5 HPF

were identified on the CD3 stained slides (as the sections for CD3 and CCR9 were adjacent to one another) and the numbers of CCR9+ cells and CD3+ cells were counted for these 5 HPF. A mean of these 5 counts was taken resulting in a mean count/5HPF for each CCR9 and CD3 within the ileum and colon for each patient. These were compared between PSC and controls. The CCR9/CD3 ratio was also determined for each of the 5 HPF, and a mean CCR9/CD3 ratio was determined and compared among groups.

CCR9+ lymphocytes were identified mainly within the lamina propria and not in the intraepithelial compartment. The staining for CCR9 within the ileum and colon was not as intense as it was for CD3, but nevertheless was dark enough to discern positive cells (see *Figure 3-10*). CCR9 staining was predominantly cytoplasmic, often with punctate cytoplasmic areas, as opposed to CD3 staining which was mainly membranous. There was very mild background staining of the epithelial cells on CCR9 staining. Intriguingly, there were lower counts of CCR9 within the ileum in all groups as compared with the colon, despite the literature showing an abundance in the small bowel and my flow cytometry findings. It must be borne in mind, however, that there has not been a description of CCR9 localisation within the human body previously using IHC. It is possible that CCR9+ T-cells are recruited to the colon just as prolifically (if not more) than the ileum, but internalisation of the receptor on lymphocyte recruitment may cause false negative results on surface antibody staining in flow cytometry experiments. Furthermore, there is a relative abundance of lymphocytes within the ileum compared with the colon, which can lead to an amplified signal on transcription analysis of the ileum.

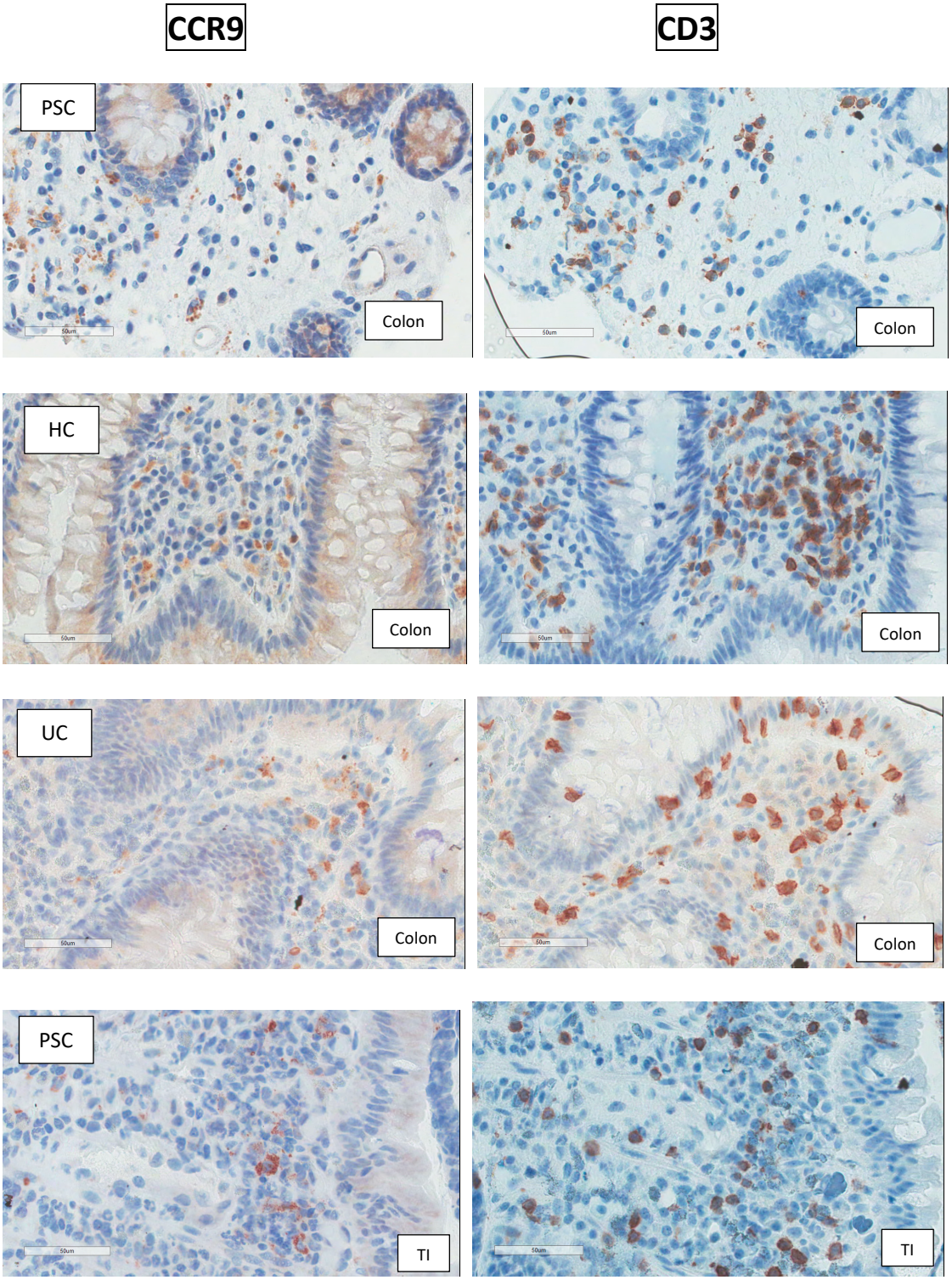


Figure 3-10 - Representative high power field views of IHC for CCR9 and CD3 of ileal and colonic samples

FFPE sections from the ileum and colon were obtained from patients with PSC/UC, UC and HC. Sections underwent IHC for CCR9 and CD3. These representative HPF mages demonstrate the paired CCR9 stained images on the left and CD3 stained images on the right from the colon (top three rows) and terminal ileum (bottom row). There was some low intensity background epithelial staining on the CCR9 images, but CCR9+ lymphocytes could still be delineated. FFPE, formalin fixed and paraffin embedded; HC, healthy controls; HPF, high power field; PSC, primary sclerosing cholangitis; TI, terminal ileum; UC, ulcerative colitis.

Similar to the flow cytometry results, there was a numerically lower mean CCR9 count/5HPF within the PSC/UC ileum compared with UC ileum (3.4 ±SEM 1.1 vs. 6.0 ±1.3) but it was not to statistical significance (p=0.212, see *Figure 3-11*). There was no difference between PSC and HC ileum. The ileal CD3 counts were much higher than the CCR9 counts, and similar among the three cohorts.

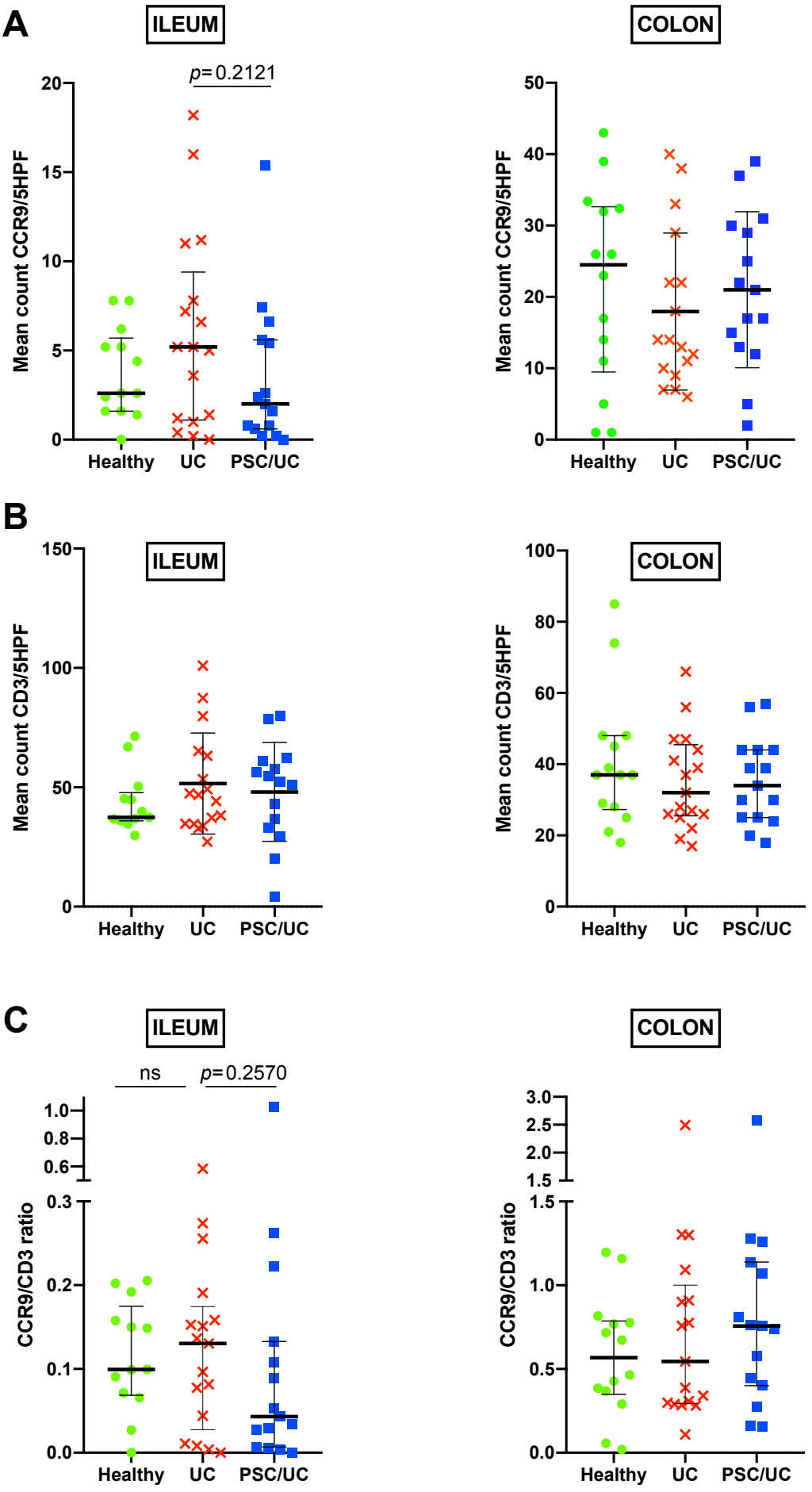
When analysing the median CCR9/CD3 ratio, again this was lower in the PSC ileum (0.043 [IQR0.007-0.133]) compared to the UC ileum (0.130, p=0.257 [IQR 0.027-0.175]), but not to statistical significance. These figures are akin to 4.3% versus 13.0%. Numerically, the median CCR9/CD3 ratio was lower in PSC compared to HC (0.099 [IQR 0.069-0.175]), but again not to statistical significance (p=0.976).

There was no difference in colonic expression of CCR9 and CD3 between PSC and controls, nor with regards to the CCR9/CD3 ratio.

Figure 3-11 - Expression of CD3 and CCR9 in the colon and ileum according to IHC analysis in PSC/UC and controls

(next page)

FFPE sections from the ileum and colon were obtained from patients with PSC/UC, UC and HC. Sections underwent IHC for CCR9 and CD3. The number of lymphocytes positive for CD3 and CCR9 respectively was counted, and the mean/5HPF was determined. The mean CCR9/CD3 ratio was also determined. A, In the ileum there as a lower mean count CR9/5HPF in PSC compared with UC, but not to statistical significance. There was no difference in CCR9 counts within the colon. B, With regards to the mean count CD3/5HPF, there was no difference between PSC and controls within the ileum of colon. C, Within the ileum there was numerically a lower CCR9/CD3 ratio in PSC compared with UC, but not to statistical significance. There was no difference within the colon. The median values ±IQR are shown. Comparisons between PSC and each individual control (UC and HC) was made via unpaired t-tests and Mann Whitney tests depending on distribution of variables. FFPE, formalin fixed and paraffin embedded; HC, healthy controls; HPF, high power field; PSC, primary sclerosing cholangitis; UC, ulcerative colitis.


A
ILEUM
p=0.2121
Mean count CCR9/5HPF
20
15
10
5
0
Healthy
UC
PSC/UC
COLON
Mean count CCR9/5HPF
50
40
30
20
10
0
Healthy
UC
PSC/UC
B
ILEUM
Mean count CD3/5HPF
150
100
50
0
Healthy
UC
PSC/UC
COLON
Mean count CD3/5HPF
100
80
60
40
20
0
Healthy
UC
PSC/UC
C
ILEUM
ns
p=0.2570
CCR9/CD3 ratio
1.0
0.8
0.6
0.3
0.2
0.1
0.0
Healthy
UC
PSC/UC
COLON
CCR9/CD3 ratio
3.0
2.5
2.0
1.5
1.5
1.0
0.5
0.0
Healthy
UC
PSC/UC

3.5 Discussion and summary of main findings

The principal finding in this chapter is the differences in CCR9 expression within the various tissue compartments in PSC versus controls. This is mainly found within the CD4+ population as analysed by flow cytometry, but IHC analysis did not differentiate for subsets of CD3+ T-cells.

There were proportionately less CCR9+ T-cells within the peripheral blood and ileum in patients with PSC compared with controls (particularly UC) which was supported, though not to statistical significance, by IHC data. Further corroboration of the differences in expression of CCR9 within the ileum is needed between PSC and controls. Quantitative PCR (qPCR) was undertaken by an honours masters student within our lab to explore this finding, and a trend towards lower CCR9 expression within the ileum was seen in PSC/UC compared with controls but not to statistical significance (data not shown). Samples were limited and there were some inconsistencies with the results, so the analysis cannot be relied upon, and hence it does not constitute part of this thesis. I plan to undertake an RNA sequencing experiment on RNA extracted from ileal, uninflamed colonic and inflamed colonic biopsies from patients with PSC/UC, UC, and HC to examine differential expression of CCR9 and as a hypothesis generating experiment to find other genes of interest.

The reason for this detected depletion of CCR9+ T-cells within the ileum in PSC is unclear, but, as mentioned earlier, could be due to internalisation of the receptor, potentially as a result of stimulation, or homing to a different tissue compartment elsewhere. This will be explored further within the next chapter.

4 The Phenotype of Liver-Infiltrating Lymphocytes in PSC

4.1 Introduction

4.1.1 Liver-infiltrating lymphocytes in PSC

There is a very limited number of studies pertaining to LILs in PSC. This is in part due to the rarity of the disease; partly due to a decreasing indications for patients undergoing liver biopsy for clinical purposes; and partly due to the difficulties encountered in isolating lymphocytes from liver tissue as compared with the ease with which this is done from peripheral blood. In any case, the literature is limited to only four studies over the past 14 years which have analysed LILs isolated from patients with PSC, and all are limited to end-stage disease given the LILs were obtained from explants of patients undergoing liver transplantation.[137, 97, 183, 138]

There are a handful of further studies which have analysed liver tissue for various immune markers without isolating the LILs, but again these are mostly limited to liver explants, and include only small numbers of patients.[184, 185, 140, 139] There are currently no data analysing LILs in patients with PSC who are at an earlier stage of disease, which, arguably, may be a time when inflammatory drivers are more prevalent than at the end-stage where the pathology is predominantly fibrosis.

4.1.2 Deriving LILs from liver samples

The first description of isolating LILs from a liver biopsy for analysis was from Sheila Sherlock's group at the Royal Free Hospital in London in 1977. In this study, they isolated

LILs from liver biopsies via mechanical digestion and could identify hepatocytes and various subsets of lymphocytes via microscopy.

Since then, given the small numbers of LILs derived from liver biopsies, techniques for isolating them from liver explants have been developed, with a particular interest and protocol development by a group in Birmingham, UK.[186] They published their protocol on LIL isolation (as well as DC and LSEC isolation) from liver tissue (explants) in 2010, where they used a combination of mechanical digestion and layering on a density gradient solution to isolate mononuclear cells. They note that enzymatic digestion with collagenase may affect some cell surface markers and therefore avoid collagenase when processing samples for lymphocyte analysis.

4.1.3 Fine needle aspiration of the liver

Fine needle aspiration of the liver (liver FNA) is a technique first described in 1895, and was devised as a way of obtaining cells from the liver for diagnostic analysis, but at a lower risk to the patient compared with percutaneous liver biopsy.[187] It involves using a much finer needle (22-25 Gauge) as compared with that used in a liver biopsy (typically 16 to 18 Gauge) - the higher the number of the Gauge, the narrower/thinner the needle, and therefore the lower the risk to the patient. In liver FNA, this thinner needle is inserted into the liver after local anaesthetic, in the same position one would perform a liver biopsy, and cells are aspirated back into a syringe, usually pre-filled with a solution.

In the past, it was employed mainly for diagnosis of malignant liver lesions, assessing for the presence of malignant cells within the isolated cell suspension. However, in recent times, it has been shown to be a successful method for obtaining reasonable numbers of

LILs for analysis for research purposes, particularly for immunophenotyping and gene expression analysis, with minimal to no adverse effects.[188-194]

In a recent study in Cardiff, Wales, 88 FNA liver were carried out, and obtained a mean of 37,000 lymphocytes per FNA liver for analysis, which is certainly sufficient for the adequate flow cytometric analysis.[190] They performed the sampling up to three times per week in patients, and it was very well tolerated with minimal to no complications. Some were performed as a one-off isolated procedure, and some just prior to a liver biopsy. There were no major complications noted, and there was minimal analgesia requirement (if any at all) as compared with liver biopsy.

In addition to the Cardiff experience, researchers in Rotterdam, Netherlands, have carried out in excess of 500 FNA liver purely for obtaining LIL for research purposes, and have encountered no serious complications.[192] This is quite notable, given the rate of a serious complication for percutaneous liver biopsy is 1%. Furthermore, historical series using the same method of FNA Liver of 1000 procedures reported no complications.[195]

Recently, another British group (Gill *et al.*) compared liver FNA with matched PBMC and liver biopsies in 28 patients with chronic hepatitis B and 15 patients with non-viral liver disease.[196] They found that the immune cells sampled by liver FNA contained liver-resident cells which were not present in the peripheral blood (such as CD8+ tissue resident memory T-cells) but to a lesser extent than cells derived mechanically from a core liver biopsy. Furthermore, they found enrichment of certain cells in liver FNA

compared with PBMC, such as MAIT cells, NK cells, and higher numbers of CD8+ T-cells, but not to the extent seen in liver biopsy-derived cells.

To date, since the initial study published in 2005 by a group in Rotterdam, liver FNA has been described in 11 studies for characterising liver-derived immune cells from groups in UK, the Netherlands, Germany and Sweden.[188-194, 196-199] The liver diseases characterised have been predominantly viral diseases – chronic hepatitis C, chronic hepatitis B, and human immunodeficiency virus (HIV) infection. Furthermore, the analyses performed on LILs derived from liver FNAs has been restricted largely to flow cytometry, with the exception of one study which has analysed the expression of 6 liver-specific genes by quantitative polymerase chain reaction (qPCR) in a small subset of liver FNA samples.[189] Their analysis suggested that liver FNA is made up of approximately 43% liver-related genes (implying some contamination/ cross over with peripheral blood) compared with 95% in liver biopsy analysis. Liver FNA has never been utilised for the analysis of LILs in PSC.

4.2 Aims and hypotheses

Given the gaps in knowledge of LILs in PSC, particularly at an earlier disease stage, the aims of this chapter are:

1. *Establish a safe and effective technique for sampling liver infiltrating lymphocytes for immunophenotyping in PSC.*
2. *Determine the proportion of liver-infiltrating lymphocytes which are positive for gut-homing markers* in PSC compared with diseased liver controls and healthy controls.*

3. *Determine the location spatially within the liver of gut-homing lymphocytes (if present).*

The hypothesis for this chapter is:

Gut-homing lymphocytes accumulate within the liver in PSC-IBD but not in control livers.*

*Gut-homing lymphocytes as denoted by markers of CCR9, $\alpha 4\beta 7$, and CD161

4.3 Methods

4.3.1 Obtaining liver FNA samples

I designed and obtained ethical approval for a study entitled "Analysis of liver infiltrating lymphocytes in primary sclerosing cholangitis by fine needle aspiration of the liver." This allowed me to recruit patients with PSC as well as patients with other non-immune-mediated liver diseases (liver controls [LC]) and obtain a liver FNA as well as paired PBMC. For PSC patients, these could be obtained either at the time of a liver biopsy if they were scheduled for one for clinical purposes, or they could have a study visit where they had FNA alone purely for research purposes. Liver FNAs were only obtained from LC at the time of a concomitant scheduled liver biopsy for clinical purposes. Inclusion and exclusion criteria, as well as patient information sheets, are listed in *Appendix 8.5*. Pain score (out of 10) at one hour post FNA, analgesia required post-procedure, and any adverse events were collected.

Patients with PSC (but not LC) were asked at their follow up telephone visit whether they would be willing to have a second FNA, and a small portion of patients were invited back for a second FNA.

4.3.2 Obtaining other liver samples

LILs were also obtained directly from liver resections, liver biopsies, and performing FNA upon liver resections *ex vivo*. Liver biopsies were obtained from patients with established or suspected liver disease who were undergoing a liver biopsy for clinical purposes. A second pass of the liver biopsy needle was carried out after the diagnostic liver biopsy to obtain a research sample under previously established ethics (REC Ref: 09/H0603/19).

Liver resections were obtained from patients undergoing resection of liver tumours through a collaboration with hepatobiliary surgeon, Mr Mike Silva, and through established biobank ethics (REC Ref: 16/YH/0247). The tumours being resected were usually colorectal cancer metastases, but occasionally were primary liver tumours such as adenomas, hepatocellular carcinomas (HCC) and other miscellaneous tumours. Tissue/ aspirates obtained were from healthy adjacent tissue and not from the tumour tissue.

4.3.3 Obtaining formalin fixed paraffin embedded liver biopsies

FFPE liver biopsies were obtained through existing Biobank ethics (REC Ref: 16/YH/0247 and REC Ref: 09/H1204/30). Archived diagnostic liver biopsies were obtained from consented patients among three cohorts: PSC/IBD, PSC without IBD, and LC.

4.3.4 Experimental analysis

LILs were isolated from FNAs, liver biopsies and resections as detailed in Chapter 2. They were stained with various antibody panels and analysed via flow cytometry and a portion were FACS sorted and underwent single cell RNA sequencing (scRNAseq). The adequacy of FNA as a technique to obtain LILs was assessed in comparison to other liver sampling techniques with respect to yield of various leukocyte subsets, as well as the liver-specific nature of LILs derived in comparison with paired PBMC. Safety outcomes of liver FNA were also assessed from the post-procedure clinical outcomes mentioned above. LILs from patients with PSC were compared with those from LC by both flow cytometry and scRNAseq to discern differing phenotypes of leukocytes between the two conditions, with particular reference to gut-homing markers.

FFPE liver biopsies were stained with antibodies against CD3 and CCR9 in the same manner as intestinal samples were in Chapter 3. Stained sections were scanned to create electronic images. The location of positive cells within the liver, if present, was described qualitatively. Five HPF areas (40X objective) with the highest density of CCR9 staining were determined by pathologist, Dr Eve Fryer, and matching HPFs were captured. Only lymphocytes with discretely positive staining were counted. The mean counts of CCR9+ and CD3+ cells in 5 HPF were determined respectively and compared between PSC and controls, as well as the CCR9/CD3 ratio.

4.4 Results

4.4.1 Baseline demographics

LILs were available from:

- 7 liver resections
- 7 liver biopsies
- 6 FNAs from resections
- 43 FNAs

4.4.1.1 Liver resection patients

Six of the seven patients (85.7%) were undergoing liver resection for suspected malignancy: 3 had metastatic adenocarcinoma from colorectal cancer (CRC), 2 had primary HCC with background NAFLD, and 1 had no cancer detected within the resection after having undergone adjuvant chemotherapy for CRC with metastases. The seventh patient had a wedge liver biopsy at the time of bariatric surgery for diagnostic purposes (and was classified here as a "resection" due to larger size of the tissue sample) and had NAFLD with moderate fibrosis. In fact, 5/7 patients had steatosis and/or fibrosis seen on pathology of the background liver, and so in essence these resections did not represent truly "healthy" liver. The mean age was 65.3 years (range 44.6-73.2) and 5/7 (71.4%) were male.

4.4.1.2 Liver biopsy patients

All 7 patients had PSC, and 6/7 had concurrent IBD (UC in all). Two patients were on VDZ at the time of the liver biopsy. There were no control liver biopsies. The median age was 32 years (range 23-61) and 3 patients (43%) were male.

4.4.1.3 FNA from resection patients

Of the 6 patients from whom FNAs were derived from liver resections, 2 had HCC with background NAFLD, 2 had metastatic adenocarcinoma from CRC with background NAFLD, and the other two had a haemangioma and neuroendocrine tumour respectively, both with normal background livers. Therefore, once again, 4/6 had steatosis/ diseased liver on the background liver sampled, and hence overall this cohort of samples did not represent "healthy" liver. The median age was 65 years (range 51-75), and 3/6 (50%) were male.

4.4.1.4 FNA patients

43 FNAs were taken from 38 patients – 5 patients had a second FNA due to an inadequate sample the first time. Of 43 FNAs, 12 (27.9%) were inadequate to use for data analysis. The reasons were: 5 had too few T-cells for analysis, 5 had a blood stained FNA sample, and the subsequent phenotype was too similar to the paired PBMC; and experimental error in 2 FNAs led to inadvertent discarding of the sample before analysis. 31 FNA samples were therefore included in the analysis. Each patient was only represented once.

The mean age was 46.9 years (IQR 34.0-60.9, range 21.1-73.7) and 23 (74.2%) were male. Just over half the patients had PSC (n=21) and the rest were liver controls (n=17). The liver controls were overrepresented by NASH compared with other liver diseases due to the preference for homogeneity in the RNA sequencing experiment, and also due to the fact that in this department, NASH is the most common indication for liver biopsy. There were no significant differences at baseline between the PSC cohort and LC cohort, except for the fact that more patients in the LC cohort underwent liver biopsy at the same time as the FNA, which was by virtue of the inclusion criteria. The baseline demographics are shown in *Table 4.1* and *Figure 4-1*.

Table 4.1 - Baseline demographics of patients undergoing fine needle aspiration

Variable	PSC (n=21)	LC (n=17)	P-value
Age, years, mean (±SEM, range)	44.2 (±13.1)	50.3 (±3.5)	0.183
Male gender, n (%)	14 (66.7%)	9 (52.9%)	0.509
Type of PSC, n (%) Classical PSC PSC/AIH overlap Small Duct-PSC	 17 (81.0%) 3 (14.3%) 1 (4.8%)	n/a	-
Type of IBD Ulcerative Colitis Crohn's Disease IBD-u No IBD	 17 (81.0%) 1 (4.8%) 1 (4.8%) 2 (9.5%)	n/a*	-
Type of liver disease NASH NAFLD (steatosis only) PBC ALD Non-specific changes on bx	 n/a	 10 (58.8%) 3 (17.6%) 2 (11.8%) 1 (11.8%) 1 (5.9%)	-
UDCA at baseline, n (%)	12 (57.1%)		
Concomitant liver biopsy, n (%)	5 (23.8%)	17 (100%)	<0.0001
Fibroscan LSM, kPa, median (IQR)[#]	11.5 (6.1-16.9)	10.4 (5.6-12.7)	0.500
Baseline bloods, median (IQR)			
ALP (IU/L)	166 (61-367)	116 (86 – 159)	0.437
ALT (IU/L)	55 (24-112)	56 (43-105)	0.733
Bilirubin (mmol/L)	10 (8-28)	10 (8-14)	0.244
Albumin (g/L)	37 (34-40)	39 (36-42)	0.158
INR	1.0 (0.9-1.0)	1.0 (1.0-1.0)	0.469
Haemoglobin (g/L)	141 (133-156)	137 (130-150)	0.318

*Two LC patients had IBD – one had IBD-u and was on VDZ, and one had CD and had insufficient cells in FNA for analysis. #Fibroscan LSM was available for 28/38 patients. AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine transaminase; bx, biopsy; IBD, inflammatory bowel disease; IBD-u; inflammatory bowel disease-unspecified; INR, international normalised ratio; LC, liver controls; LSM, liver stiffness measurement; n/a, not applicable; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; UDCA, ursodeoxycholic acid; VDZ, vedolizumab.

4.4.2 Safety analysis of FNA as a liver sampling technique

Of the 43 FNAs performed, 22 were performed concomitantly with a liver biopsy, and 21 were performed as a single FNA procedure. The median pain score marked out of 10 at one hour was higher in the liver biopsy/FNA cohort (1.5 [IQR 0-6.8]) than in the FNA only cohort (0.0 [IQR 0.0-1.5], p=0.006, see *Figure 4-1*). Analysing the pain score a different way, 73.7% of patients in the liver biopsy/FNA group experienced pain post-procedure (pain score ≥1) compared with only 25.0% in the FNA only group (p=0.012).

One patient (4.8%) required analgesia in the FNA only group versus 9 patients (40.9%) in the liver biopsy/FNA group (p=0.005). Of those that required analgesia, most only required paracetamol only (7/10), though 3 patients required subsequent opiate analgesia (oral codeine in one patient in PSC cohort, oral codeine in one patient in LC cohort, and intravenous opiates in a second patient in LC cohort).

One serious adverse event occurred within the liver biopsy/FNA group. One patient with PSC/UC experienced significant pain and was found on CT to have a small contained intrahepatic haematoma. She was haemodynamically stable. She was admitted to hospital and observed overnight, and discharged the following day with no long-term sequelae.

Qualitatively, FNA was well received by patients. The majority of patients were willing to undergo FNA after reading the patient information sheet, and the majority of patients were happy to have a second FNA if required. We asked patients with PSC only if they were happy for a second FNA. Of 17 patients asked, 15 patients (88%) were happy to be contacted to undergo a repeat FNA.

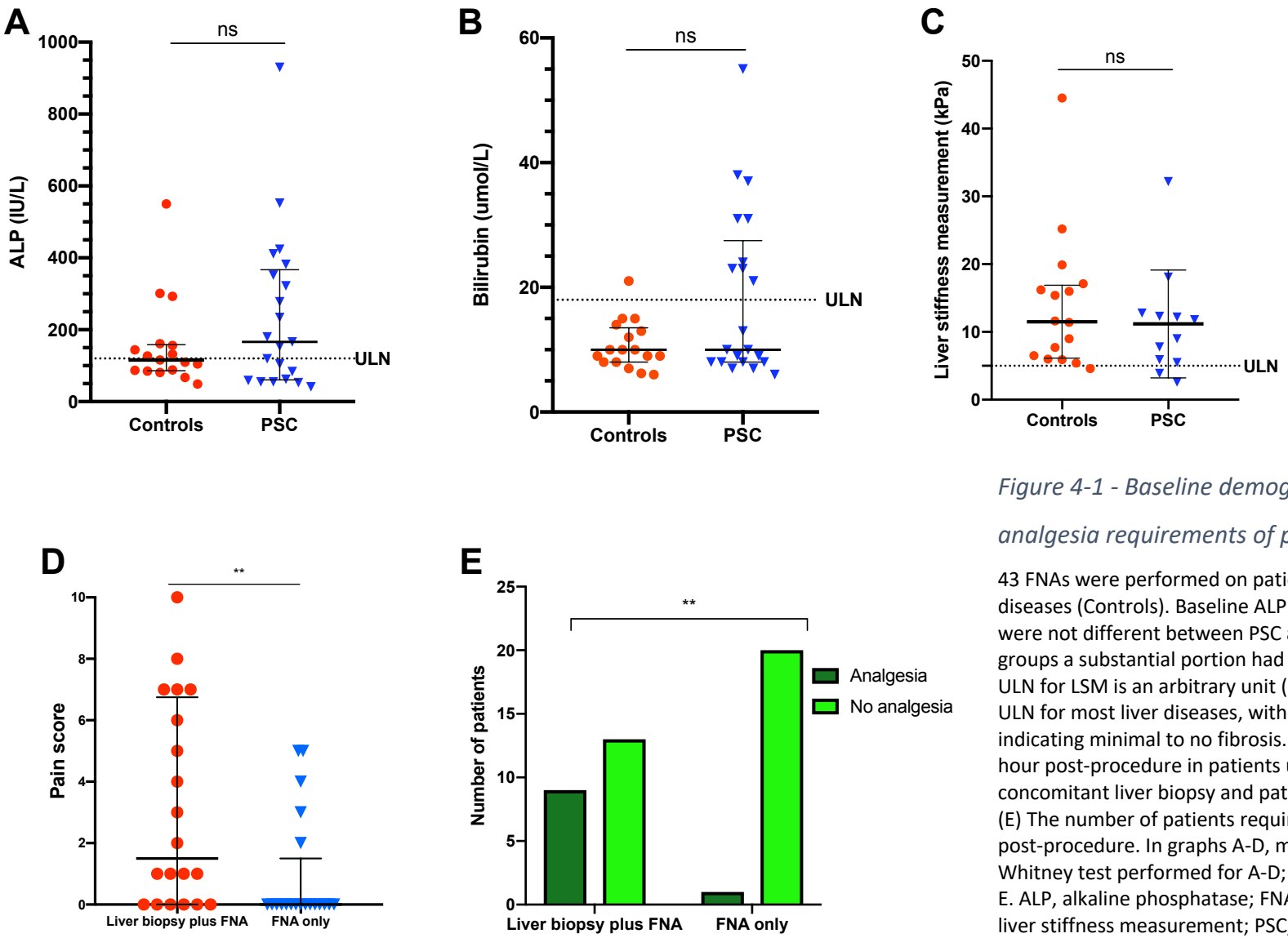


Figure 4-1 - Baseline demographics, pain score and analgesia requirements of patients undergoing FNA.

43 FNAs were performed on patients with PSC and other liver diseases (Controls). Baseline ALP (A), bilirubin (B) and LSM (C) were not different between PSC and Controls, although in both groups a substantial portion had values above the ULN. The ULN for LSM is an arbitrary unit (5kPa) which is consistent with ULN for most liver diseases, with values below this threshold indicating minimal to no fibrosis. (D) Pain score out of 10 at one hour post-procedure in patients undergoing FNA with concomitant liver biopsy and patients undergoing FNA alone. (E) The number of patients requiring analgesia during recover post-procedure. In graphs A-D, median±IQR are depicted. Mann Whitney test performed for A-D; Chi-square test performed for E. ALP, alkaline phosphatase; FNA, fine needle aspirate; LSM, liver stiffness measurement; PSC, primary sclerosing cholangitis; U:N, upper limit of normal.
ns=not significant, i.e. p>0.05. **p=0.001-0.01.

Taken together, these data suggest that FNA is a safe technique which patients are willing to undergo to sample LILs. FNA alone had a lower pain score post-procedure than paired with liver biopsy, was usually associated with no post-procedure pain at all, and it was not associated with any serious adverse events, except in the setting of a concomitant liver biopsy.

4.4.3 Adequacy of FNA and different liver sampling techniques for obtaining liver infiltrating lymphocytes

4.4.3.1 Yield of lymphocytes from FNA and other liver sampling techniques

All sampling techniques, including FNA, delivered adequate numbers of lymphocytes for analysis via flow cytometry. The gating strategy for identifying lymphocytes and various subsets of interest are shown in *Figure 4-2*. t-Distributed Stochastic Neighbour Embedding (t-SNE) was able to reveal various clusters and cell subsets (see *Figure 4-3*.)

The median yield of lymphocytes obtained was greatest numerically for those sampled from liver resections directly, with lower amounts using the other three sampling techniques: Resection: 57,057 lymphocytes (IQR 16,227 to 392,624); biopsy: 11,804 (IQR 10,435 to 24,502); FNA from resection: 19,412 (IQR 8333 to 50,800); FNA: 12,333 (IQR 6,392 to 24,205; p=0.020 for FNA vs. resection, other comparisons not significant). This was also true for the median yield of CD3+ T-cells obtained, though none of the comparisons were statistically significant. The median yield of CD3+ T-cells from FNA was 7,547 T-cells (IQR 4,456 to 15,910). The number of CD4+ and CD8+ T-cells was actually similar among all the sampling techniques. Notably, there was significant variation in the yield of these cell subsets obtained within each liver sampling techniques as seen in *Figure 4-4*.

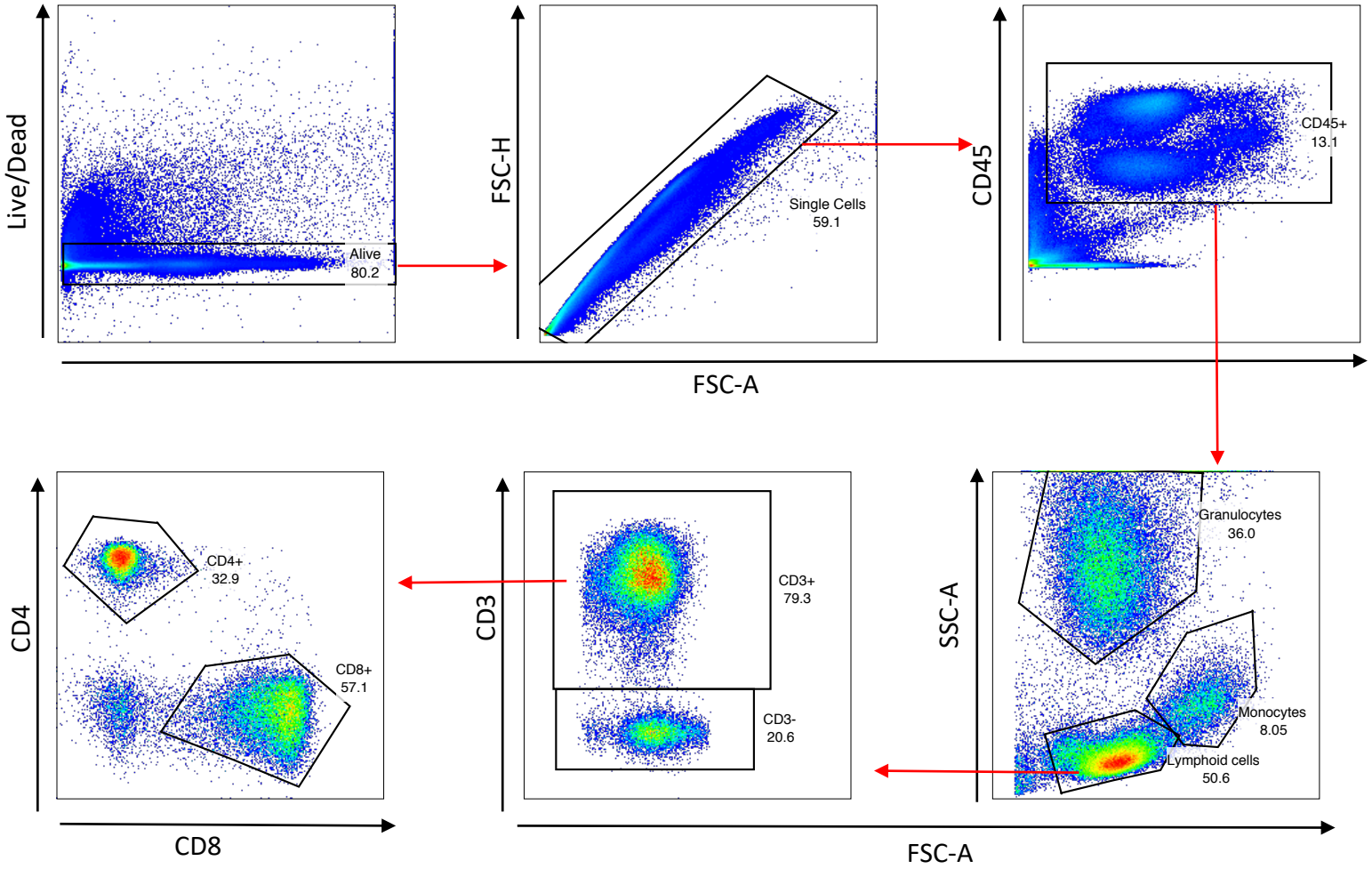


Figure 4-2 - Gating strategy of various lymphocyte subsets on liver FNA sample.

Representative scatter plots of a liver FNA sample. Cells were gated first on live cells, then singlets, followed by CD45+ cells to detect the haematopoietic component. Subsequently, through forward- versus side-scatter, lymphocytes were detected and gated, and CD3+ cells were selected. CD3+ T-cells could then be gated on either CD4 or CD8 expression for analysis. The red arrows indicate the gate taken forward to the next scatter plot. A, area; FNA, fine needle aspirate; FSC, forward scatter; H, height; SSC, side scatter.

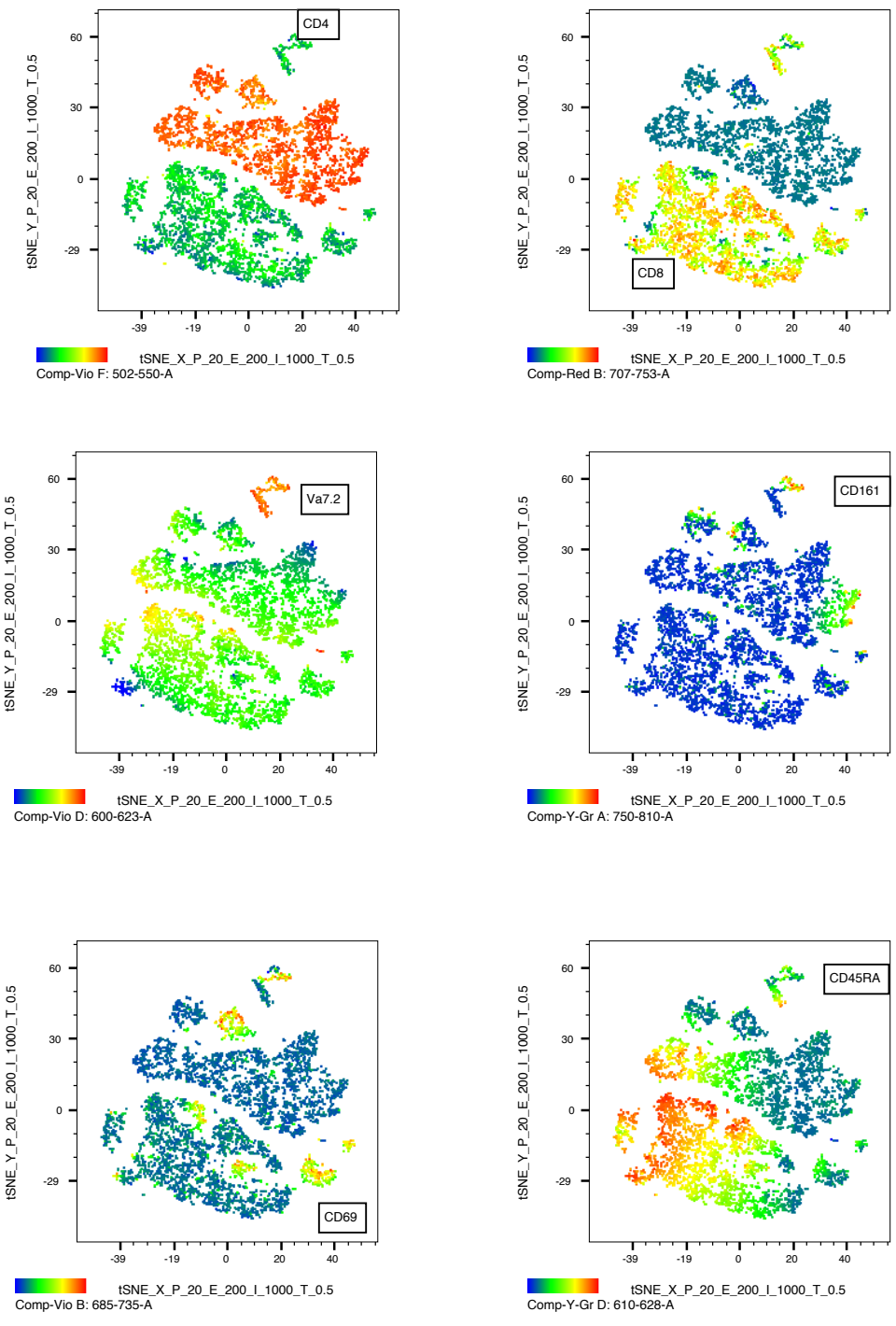


Figure 4-3 – t-SNE plots showing differential expression of various cell surface markers on CD3+ T-cells from a liver FNA among different clusters.

t-SNE is a non-linear dimensionality reduction technique to visualise high dimension data in a low, two or three dimension setting. These are representative plots where t-SNE has been applied to gated CD3+ T-cells from a liver FNA to separate the cells into various clusters, as determined by the 13 antigen markers within the flow cytometry panel. The expression of 6 of these markers is shown via colour differentiation with the heat map indicated below (blue – lowest expression, red highest expression). FNA, fine needle aspirate; t-SNE, t-distributed stochastic neighbour embedding.

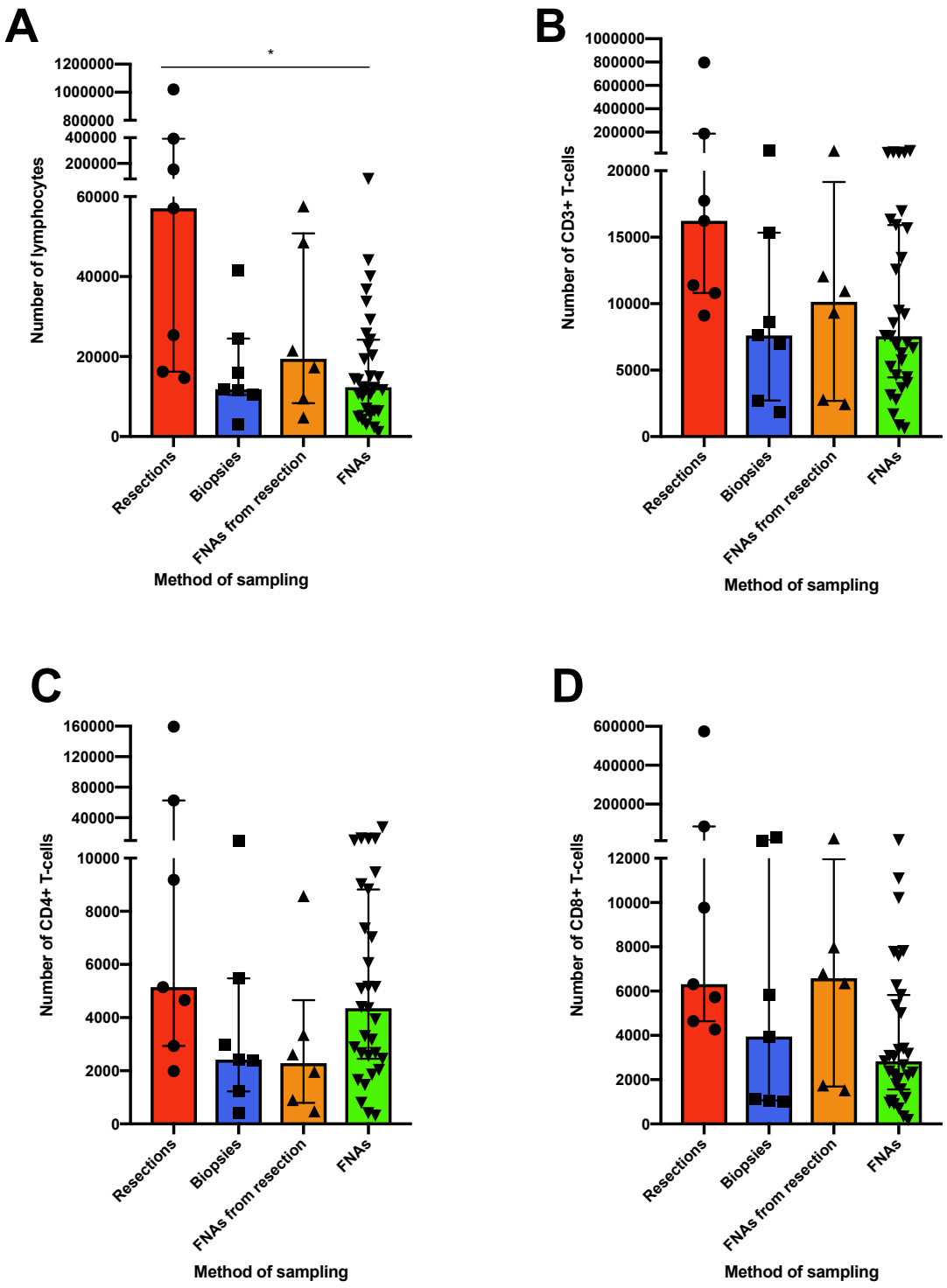


Figure 4-4 - Yield of lymphocytes and subsets among differing liver sampling techniques

Lymphocytes were obtained via four different sampling techniques: directly from resections, from liver biopsies, FNA *ex vivo* from liver resections and FNAs. The yield of lymphocytes and the various lymphocyte subsets are compared among techniques. Unless stated, all comparisons were not significant. The graphs represent the yield, as determined by flow cytometry, of: A, lymphocytes, B, CD3+ T-cells; C, CD4+ T-cells; and D, CD8+ T-cells. The median and IQR are depicted. FNA, fine needle aspirate. Kruskal Wallis test with Dunn's multiple comparisons test was applied. *=$p<0.05$.

4.4.3.2 Presence of tissue-specific LIL subsets in FNA and other sampling techniques.

In peripheral blood there is usually a greater proportion of CD4+ T-cells compared with CD8+ T-cells among the CD3+ T-cell subset, whereas this is usually reversed within the tissue compartment. Whilst not specifically tissue/liver-specific, one way of analysing whether CD3+ T-cells are truly liver-derived is to look for a greater proportion of CD8+ T-cells and a lower proportion of CD4+ T-cells.

There were less CD4+ T-cells derived from liver FNA compared with paired PBMC, and, likewise, a greater proportion of CD8+ T-cells, indicating that at least a portion of CD3+ T-cells derived from FNA were likely liver-derived (see *Figure 4-5*). This was also true for CD3+ T-cells derived from liver biopsies and from FNAs from resections. Paired blood samples were not available for LILs derived directly from resections. When comparing the proportion of CD4+ and CD8+ T-cells derived from the four different liver sampling techniques, there were more CD4+ T-cells and less CD8+ T-cells among CD3+ T-cells derived from FNAs compared with other liver sampling techniques, particularly compared with FNAs from resections.

Similarly, there was a higher proportion of memory T-cells (CD45RA-) among liver-derived CD4+ and CD8+ T-cells compared with paired peripheral blood CD4+ and CD8+ T-cells, indicating the presence of liver-derived T-cells in FNAs and other types of liver samples (see *Figure 4-6* and *Figure 4-7*). The proportion of memory T-cells was numerically less in cells derived from FNA compared with other sampling techniques.

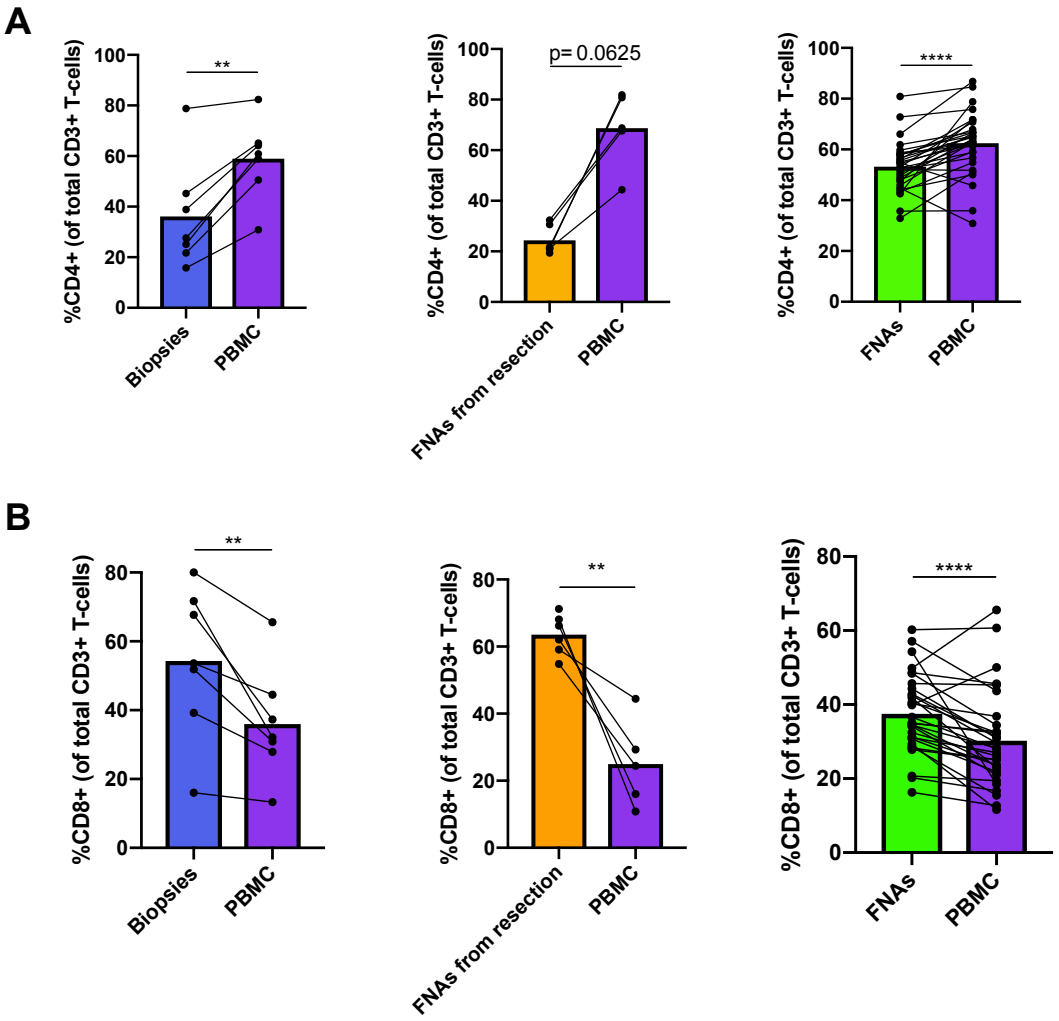


Figure 4-5 - The proportion of liver-derived and peripheral CD3+ T-cells which are CD4+ and CD8+ positive among differing liver sampling techniques

LILs were isolated from patients from liver resections, liver biopsies, FNAs from liver resections and FNA of the liver. Paired PBMC were obtained for three of these subsets of patients. Cells were stained for various cell surface markers and via flow cytometry, analysis of the proportion of CD3+ T-cells which were CD4+ (A) and CD8+ (B) was determined. The proportions were compared between LILs and paired PBMC (A&B). LILs had a lower proportion of CD4+ T-cells and higher proportion of CD8+ T-cells compared with paired PBMC. C, comparison among differing liver sampling techniques of CD4+ proportions (upper graph) and CD8+ proportions (lower graph) among CD3+ T-cells. In all graphs, spaghetti plots represent each individual patients' paired samples, and the mean is depicted by the bar. Paired T-test or Wilcoxon matched-pairs signed rank test was applied in A&B to normally and non-normally distributed samples respectively. Kruskal Wallis test and Dunn's multiple comparison test was applied in C. FNA, fine needle aspirate; LIL, liver infiltrating lymphocytes; PBMC, peripheral blood mononuclear cells.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

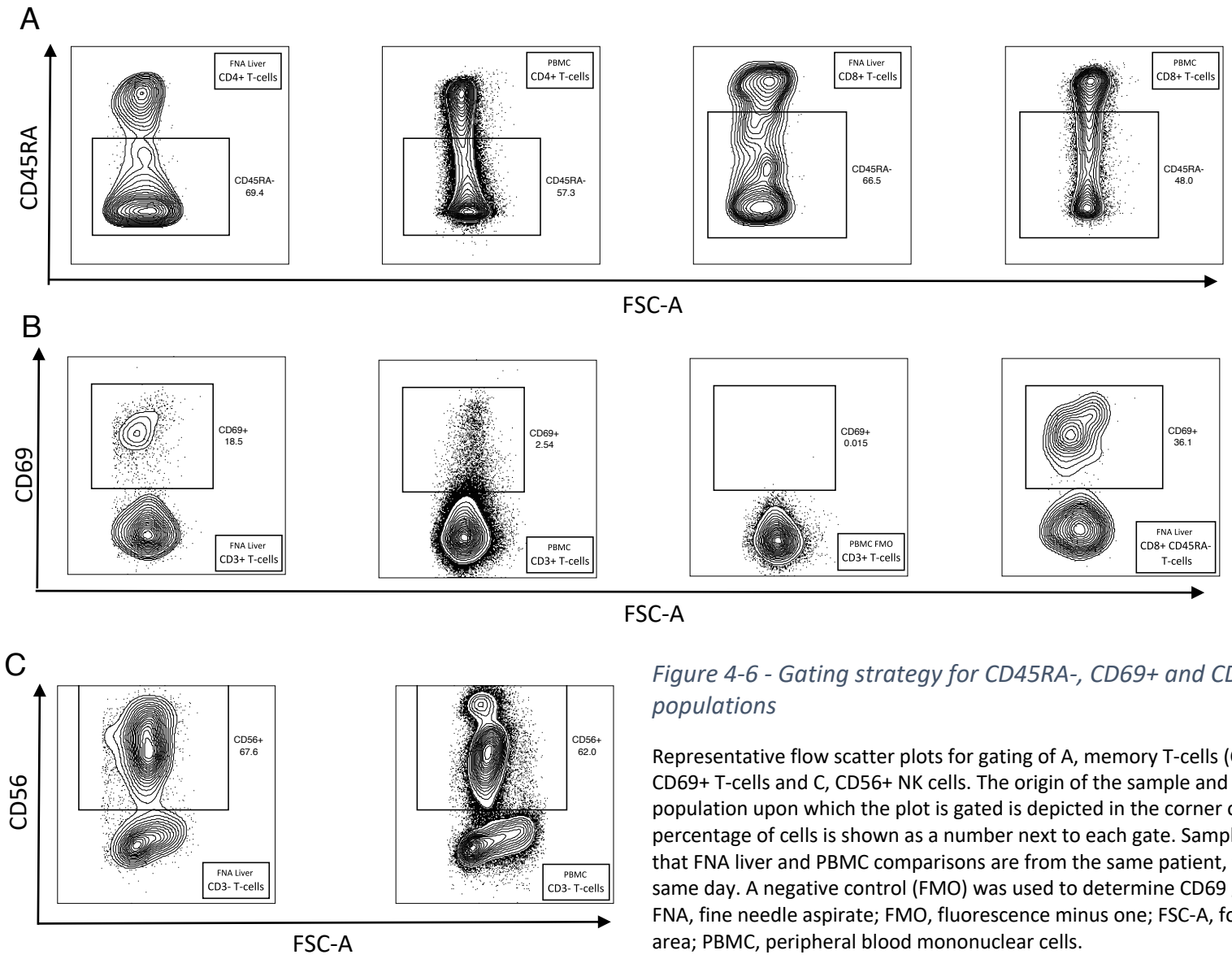


Figure 4-6 - Gating strategy for CD45RA-, CD69+ and CD56+ populations

Representative flow scatter plots for gating of A, memory T-cells (CD45RA-); B, CD69+ T-cells and C, CD56+ NK cells. The origin of the sample and the cell population upon which the plot is gated is depicted in the corner of each plot. The percentage of cells is shown as a number next to each gate. Samples are paired, in that FNA liver and PBMC comparisons are from the same patient, taken on the same day. A negative control (FMO) was used to determine CD69 gating in (B). FNA, fine needle aspirate; FMO, fluorescence minus one; FSC-A, forward scatter-area; PBMC, peripheral blood mononuclear cells.

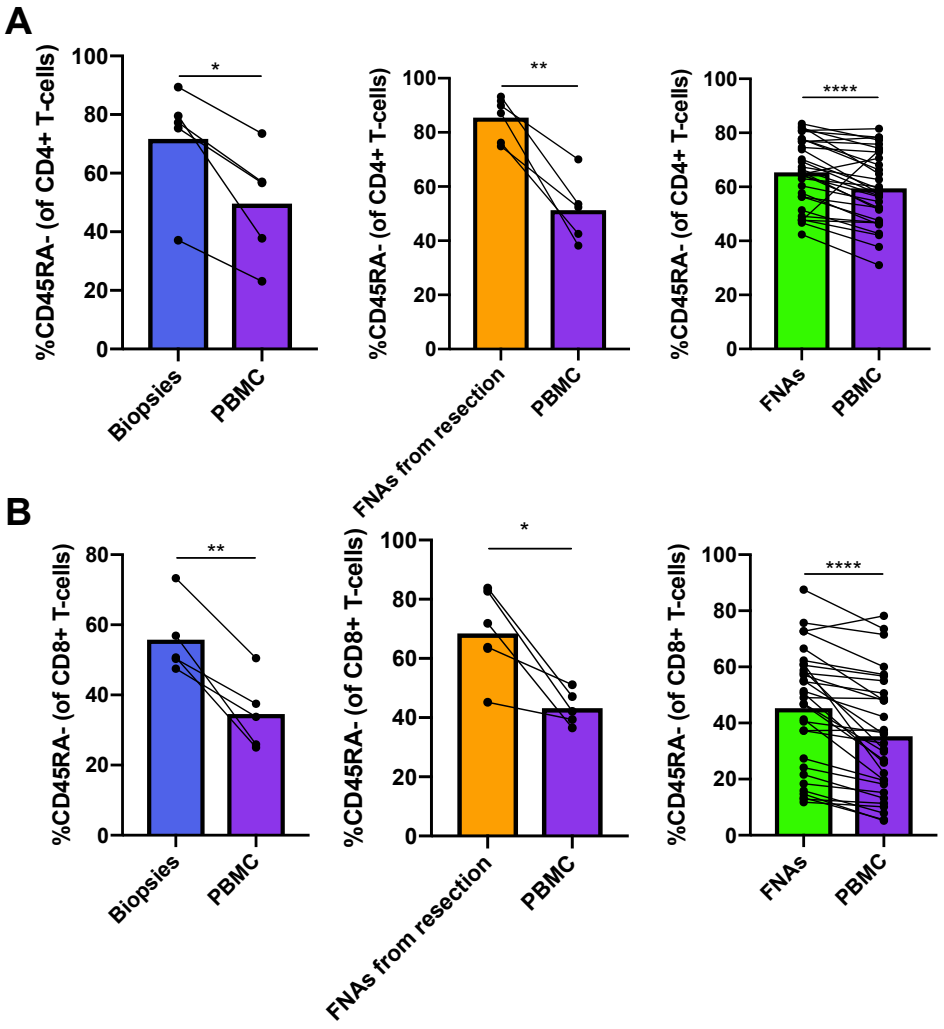


Figure 4-7 - *The proportion of liver-derived and peripheral CD4+ and CD8+ T-cells which are memory T-cells among differing liver sampling techniques.*

LILs and PBMC were obtained as in *Figure 4-5* and gated on either CD4+ T-cells (A) and CD8+ T-cells (B). The proportion of cells which were memory T-cells (CD45RA-) were compared between liver and matched peripheral blood T-cells (A&B) and among T-cells derived from differing liver sampling techniques (C, upper panel, CD4+ T-cells; lower graph, CD8+ T-cells). There was a higher proportion of memory T-cells within liver populations compared with peripheral blood populations. In all graphs, spaghetti plots represent each individual patients' paired samples, and the mean is depicted by the bar. Paired T-test or Wilcoxon matched-pairs signed rank test was applied in A&B to normally and non-normally distributed samples respectively. Kruskal Wallis test and Dunn's multiple comparison test was applied in C. FNA, fine needle aspirate; LIL, liver infiltrating lymphocytes; PBMC, peripheral blood mononuclear cells. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

Two other cell subsets of interest that were analysed were CD69+ T-cells and NK cells (CD3- CD56+), as depicted in *Figure 4-6*. Both are known to be elevated in the liver as compared with the peripheral blood, and this was found to be true for all liver sampling techniques (see *Figure 4-8*). As per the earlier liver-nature cell subtypes evaluated, the lowest proportion numerically of CD69+ T-cells (among CD3+ T-cells) and CD56+ NK cells (among CD3- cells) was seen in FNA samples as compared with other liver sampling techniques, but nevertheless, these proportions were higher than seen in peripheral blood. Furthermore, when analysing FNA samples versus paired PBMC, there were more CD69+ T-cells among CD4+ and CD8+ memory T-cells in particular, as well as among NK cells.

Pembroke *et al.* demonstrated via FNA that the proportion of NK cells (CD3- CD56+) which were CD16+ was lower in the liver compared with the peripheral blood (the latter of which are almost all CD16+).[190] I was able to show this in our FNA samples as compared with paired peripheral blood samples (see *Figure 4-10*).

Figure 4-8 - the proportion of CD69+ cells among varying leukocyte subsets in the liver and peripheral blood.

LILs and PBMC were obtained as in *Figure 4-5* and gated on A and C, CD3+ T-cells and B, CD4 memory T-cells (left), CD8+ memory T-cells (middle) and CD3- CD56+ NK cells (right). A and B, the proportion of each leukocyte subset which were CD69+ was compared between liver-derived samples and matched peripheral blood samples for the various liver sampling techniques. There was a greater proportion of CD69+ cells among all leukocyte subsets in the liver compared with the peripheral blood with all the liver sampling techniques. The exception was cells derived from liver biopsies, as only 2 of the samples were analysed with a flow cytometry antibody panel which contained CD69. C, the proportion of CD3+ T-cells which were CD69+ was compared among the differing liver sampling techniques. In all graphs, spaghetti plots represent each individual patients' paired samples, and the mean is depicted by the bar. Paired T-test or Wilcoxon matched-pairs signed rank test was applied in A&B to normally and non-normally distributed samples respectively. Kruskal Wallis test and Dunn's multiple comparison test was applied in C. FNA, fine needle aspirate; LIL, liver infiltrating lymphocytes; PBMC, peripheral blood mononuclear cells. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

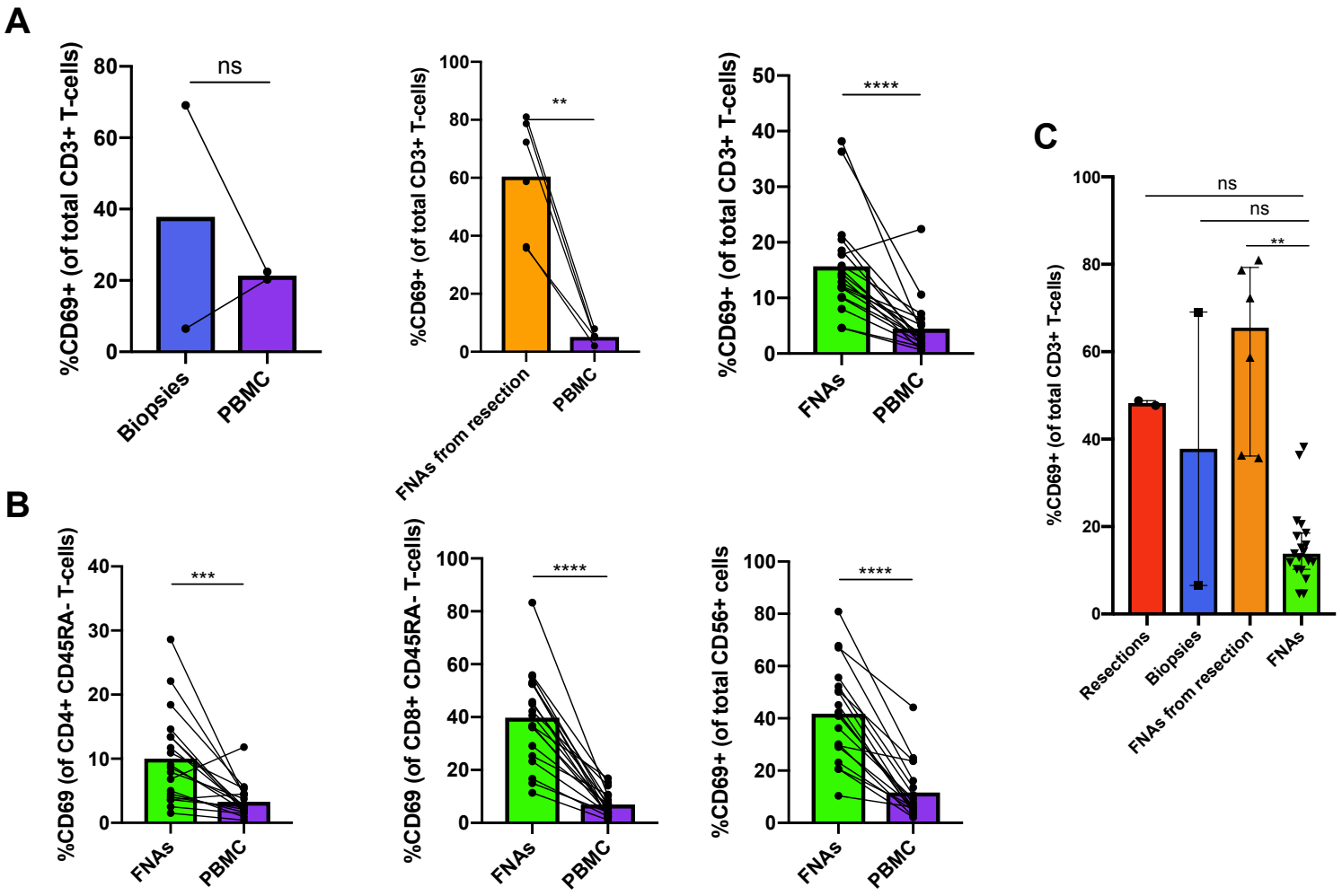


Figure 4-9 - the proportion of CD69+ cells among varying leukocyte subsets in the liver and peripheral blood.

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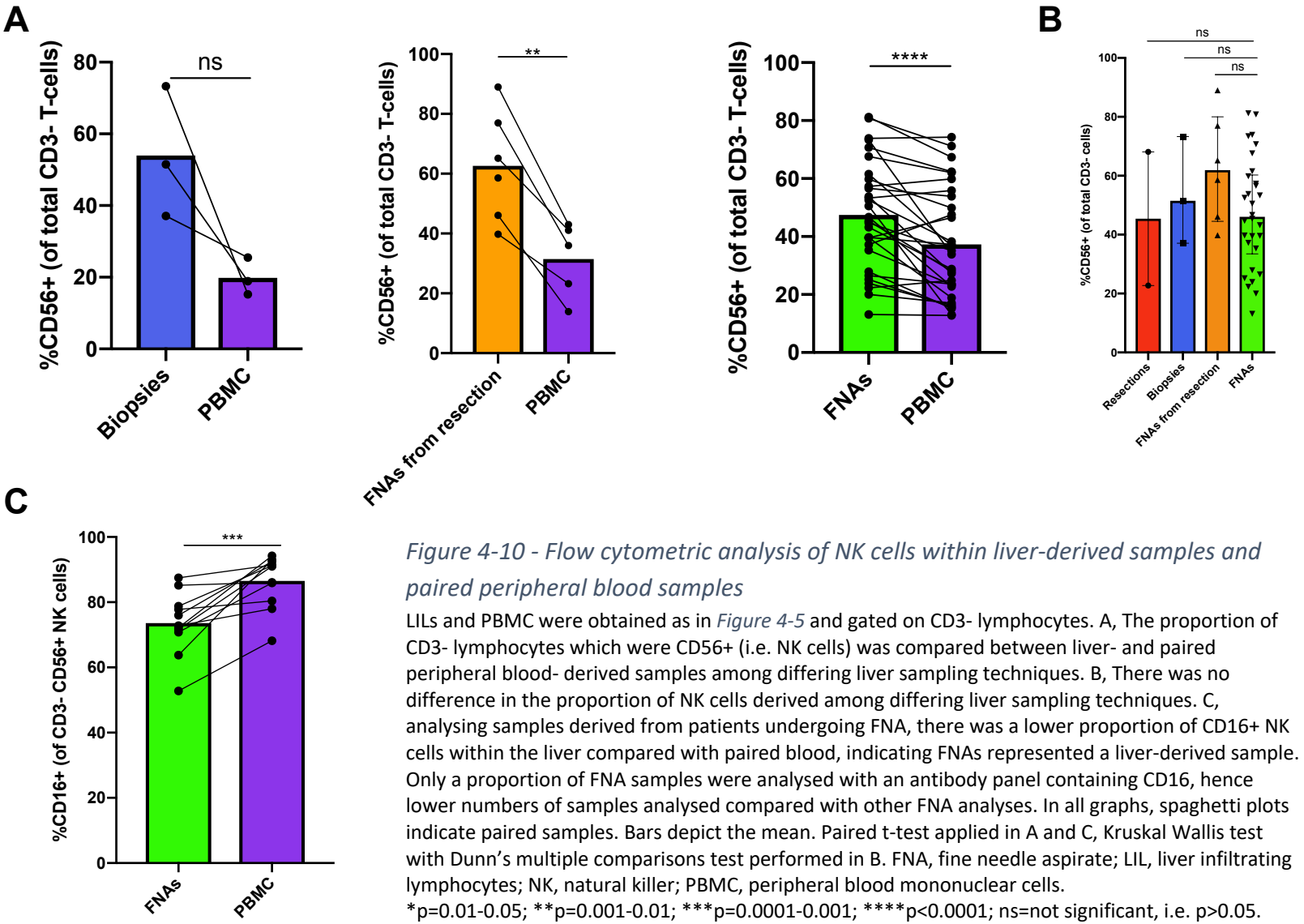


Figure 4-10 - Flow cytometric analysis of NK cells within liver-derived samples and paired peripheral blood samples

LILs and PBMC were obtained as in *Figure 4-5* and gated on CD3- lymphocytes. A, The proportion of CD3- lymphocytes which were CD56+ (i.e. NK cells) was compared between liver- and paired peripheral blood- derived samples among differing liver sampling techniques. B, There was no difference in the proportion of NK cells derived among differing liver sampling techniques. C, analysing samples derived from patients undergoing FNA, there was a lower proportion of CD16+ NK cells within the liver compared with paired blood, indicating FNAs represented a liver-derived sample. Only a proportion of FNA samples were analysed with an antibody panel containing CD16, hence lower numbers of samples analysed compared with other FNA analyses. In all graphs, spaghetti plots indicate paired samples. Bars depict the mean. Paired t-test applied in A and C, Kruskal Wallis test with Dunn's multiple comparisons test performed in B. FNA, fine needle aspirate; LIL, liver infiltrating lymphocytes; NK, natural killer; PBMC, peripheral blood mononuclear cells.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

Finally, as mentioned earlier, it has been shown that there is a higher proportion of MAIT cells within the liver compared with peripheral blood. The data for CD161+ T-cells, particularly CD161+ CD4+ T-cells, in the liver are lacking. I found, both in FNAs from liver resections (non-significant) and liver FNAs, that there was a higher mean proportion of CD161+ CD4+ T-cells in the liver compared with paired peripheral blood: FNAs from resection 36.2% ±SEM12.5 vs. 14.2% ±2.1, p=0.169; FNAs 26.1% ±SEM1.3 vs. 22.0% ±1.4, p=0.008. I also found a greater median proportion of MAITS among CD3+ T-cells (data not shown) and among CD8+ T-cells in liver-derived samples from FNAs from resections (numerical only) and FNAs: FNAs from resection 18.1% (IQR 5.0-35.9) vs. 5.0% (IQR 2.0-9.1, p=0.133, proportions are of CD8+ T-cells); FNAs 3.7% (IQR 2.1-14.3) vs. 2.3% (IQR 1.0-4.9, p=0.0007, proportions are of CD8+ T-cells).

Taking all these data together, FNAs are distinct from paired PBMC in that they have:

- Higher proportions of:
    - CD8+ T-cells
    - Memory T-cells
    - CD69+ cells (among CD3+, memory CD4+ and memory CD8+ T-cells and NK cells)
    - CD161+ CD4+ T-cells
    - MAIT cells
    - NK cells
- Lower proportions of:
    - CD4+ T-cells
    - CD16+ NK cells

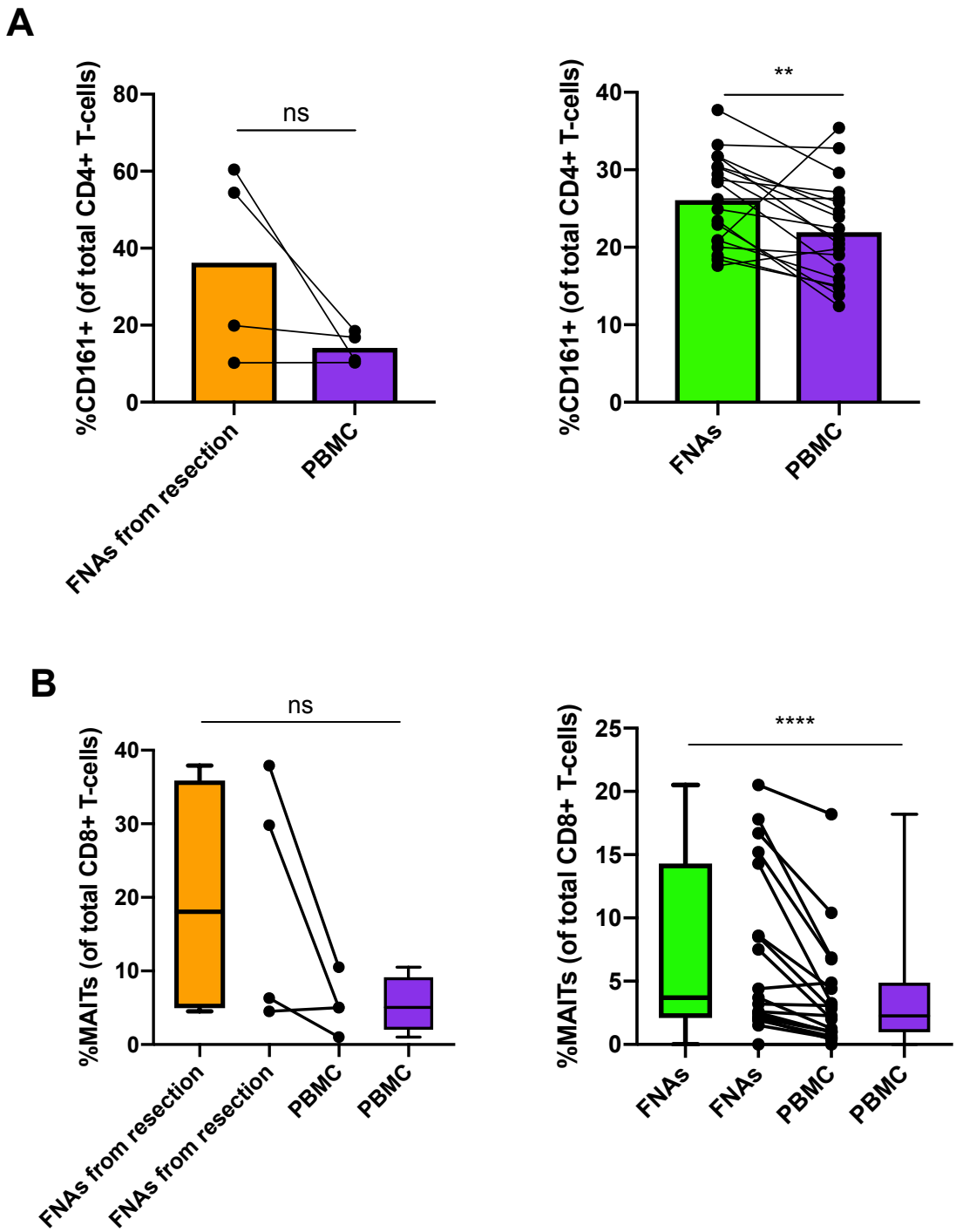


Figure 4-11 - Flow cytometric analysis CD161+ cells in liver-derived samples and peripheral blood

LILs and paired PBMC were obtained as in *Figure 4-5* and gated on CD4+ and CD8+ lymphocytes. A, the proportion of CD4+ T-cells which were CD161+ was compared between liver-derived cells and paired peripheral blood cells. There was a greater proportion of CD161+ CD4+ T-cells in liver FNA samples compared with paired PBMC samples. B, The proportion of CD8+ T-cells which were MAIT cells (CD161++ Vα7.2+) was compared between liver- and peripherally derived samples. There was a higher proportion of MAITs in liver FNA samples compared with PBMC. In A&B, spaghetti plots represent paired patient samples. In A, the mean is depicted, and paired t-test applied. In B, median±IQR is shown, and Wilcoxon test applied. FNA, fine needle aspirate; LIL, liver infiltrating lymphocytes; MAITs, mucosa associated innate-like T-cells; PBMC, peripheral blood mononuclear cells.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

These above data suggest that the technique of FNA does indeed sample a liver-derived population. However, when comparing FNA as a liver lymphocyte sampling technique with other sampling techniques described here, the proportion which are liver-derived (or "tissue resident") is not as great among FNAs. FNAs from resections (which is in essence FNA *ex vivo*) in particular had very high proportions of cells more likely to be "tissue resident" such as CD69+ T-cells and memory T-cells. This is interesting in itself, and does not necessarily mean that FNA *in vivo* is not as accurate at reflecting the liver immune cells as the other techniques.

Whilst a possible explanation could be that the FNA may be contaminated with peripheral blood aspirated during the procedure, this is less likely to be the full explanation when the amounts of blood staining seen peri-procedurally were miniscule (of the order of 100-500μL), and the amount of T-cells derived was far higher than would be expected from such a small amount of peripheral blood. A more likely explanation is that sampling the liver *in vivo* with FNA sampled not only liver-resident leukocytes, but also liver sinusoidal lymphocytes, where *ex vivo* sampling (as in the other three methods) may lead to a distinct lack of sinusoidal blood and therefore less sinusoidal leukocytes.

It may be argued that sinusoidal leukocytes are just as important to analyse as liver-resident leukocytes due to the known interactions and subsequent downstream signalling between various leukocyte subsets and LSECs.

Hence, these data show that FNA of the liver (*in vivo*) is an adequate technique to sample the liver immune microenvironment, with various components indicating the cell

population is liver-derived. Comparisons with traditional *ex vivo* sampling techniques show that FNA samples a unique liver immune population.

4.4.4 Gut-homing surface protein expression on T-cells via flow cytometry in PSC vs. LC

Given the slight differences in the immune populations obtained between FNAs and other liver sampling techniques, I decided to only compare different disease phenotypes of T-cells derived from FNA only rather than mix together the liver-sampling techniques. Given that healthy controls could only be derived from resections or FNAs from resections, PSC samples were not compared with healthy controls, but LC only.

Furthermore, I considered comparing the CD3+ CD69+ T-cells derived from FNAs, rather than the whole CD3+ subset, but did not for two reasons. Firstly, I considered the sinusoidal T-cells to be important in the comparisons being made, and selecting out the CD69+ T-cells (and potentially the more tissue-resident T-cells) may exclude this important subset. Secondly, the yield of CD69+ T-cells was too low to then make meaningful comparisons among further downstream subsets. The median number of CD4+ CD69+ T-cells was 213 cells (IQR 144-272) and for CD8+ CD69+ T-cells it was 506 cells (IQR 364-1379).

There were three patients within the cohort of 31 patients who were on VDZ at the time of FNA (2 in PSC cohort, 1 in LC cohort), and these patients were excluded from the subsequent analyses in case of an effect on the phenotype of hepatic lymphocytes present.

4.4.4.1 CCR9+ expression

There was no difference in the mean proportion of hepatic CD4+ memory T-cells which were CCR9+ between PSC (4.4% ±SEM0.7) and LC (4.0% ±0.5, p=0.604, see *Figure 4-12*). This was also true among CD8+ memory T-cells. Interestingly, there was a small proportion of patients in each group who had rather higher proportions of CCR9+ T-cells among CD4+ memory T-cells. Patients in the PSC group had the highest proportion of CCR9+ CD4+ memory T-cells. The 5 patients with the highest CD4+ CCR9 expression among the LC group all had very high liver stiffness measurement (LSM) – range 16-44kPa. Of the three patients in the PSC group who had the highest CD4+ CCR9 expression only 1/3 had a LSM reading (12.2kPa). Another patient had platelets in the lower part of the normal range (176 x 10^9/L), and the third patient had a low fibrosis score on histology. In any case, a correlation could not be drawn between LSM and proportion of CD4+ memory cells which were CCR9+ (*Pearson r*=0.224, p=0.357).

4.4.4.2 β7 expression

Accordingly, there was no difference in the proportion of CD4+ and CD8+ memory T-cells which were β7+ (see *Figure 4-12*). It should be noted, however, that a substantial portion of CD4+ memory T-cells and CD8+ memory T-cells were β7+: for PSC patients, median 20.4% (IQR 15.1-26.2) of CD4+ memory T-cells and median 51.2% (IQR 40.5-60.1) of CD8+ memory T-cells. As there was no α4 antibody in the panel, it is unclear whether these cells are α4β7+, αEβ7+ or both. CD8+ CD69+ αEβ7+ T-cells within the liver have been said to be resident memory T-cells; however, they only make up a small proportion of cells. Furthermore, CD4+ T-cells rarely express αEβ7, so it is a reasonable assumption that the CD4+ T-cells analysed here as β7+ are in fact α4β7+.

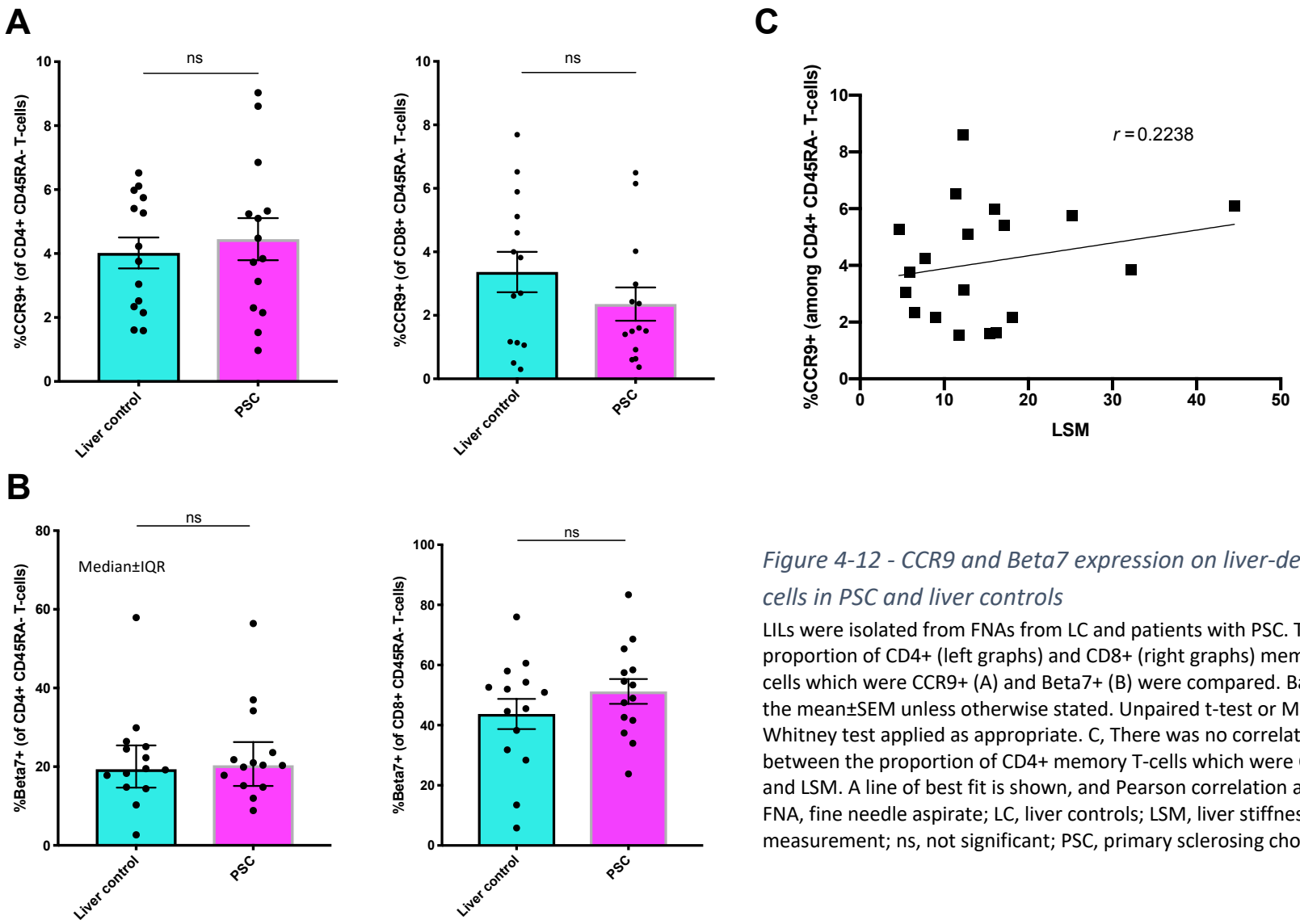


Figure 4-12 - CCR9 and Beta7 expression on liver-derived T-cells in PSC and liver controls

LILs were isolated from FNAs from LC and patients with PSC. The proportion of CD4+ (left graphs) and CD8+ (right graphs) memory T-cells which were CCR9+ (A) and Beta7+ (B) were compared. Bars depict the mean±SEM unless otherwise stated. Unpaired t-test or Mann Whitney test applied as appropriate. C, There was no correlation between the proportion of CD4+ memory T-cells which were CCR9+ and LSM. A line of best fit is shown, and Pearson correlation applied. FNA, fine needle aspirate; LC, liver controls; LSM, liver stiffness measurement; ns, not significant; PSC, primary sclerosing cholangitis.

4.4.4.3 CD161 and CD69 expression

A substantial portion of hepatic CD4+ memory T-cells were CD161+ however there was no difference between LC and patients with PSC; LC: mean 38.8% (±SEM 1.9) vs. PSC: mean 40.3% (±1.9, p=0.582, see *Figure 4-13*). There was a trend towards a higher proportion of memory CD8+ T-cells which were MAITs in the PSC group (mean 16.7% ±3.2) compared with LC (10.4% ±2.9, p=0.159). The significance of this is unclear. It is interesting that there is a cluster of patients in each group, but particularly the PSC group, who have a substantially higher proportion (e.g. >10%) of CD8+ memory T-cells which are MAITs. All the 5 patients in the PSC group with high levels of MAITs had IBD, but there were no obvious differences between these patients compared with those with lower MAIT proportions.

Whilst there was no difference in the proportion of CD4+ memory T-cells which were CD69+ between PSC and LC, there were substantially more CD69+ T-cells among CD8+ memory T-cells in patients with PSC compared with LC, mean 48.9% (±4.8) vs. 29.7% (±6.4) respectively (p=0.026, see *Figure 4-13*). This may indicate a more active inflammation within the liver and therefore more activated CD8+ T-cells in those patients with PSC compared with LC. Alternatively it may be a sign of a higher proportion of tissue resident memory (T_{RM}) CD8+ T-cells within the liver of patients with PSC, given T_{RM} are denoted by CD69 and CD103 expression.[200]

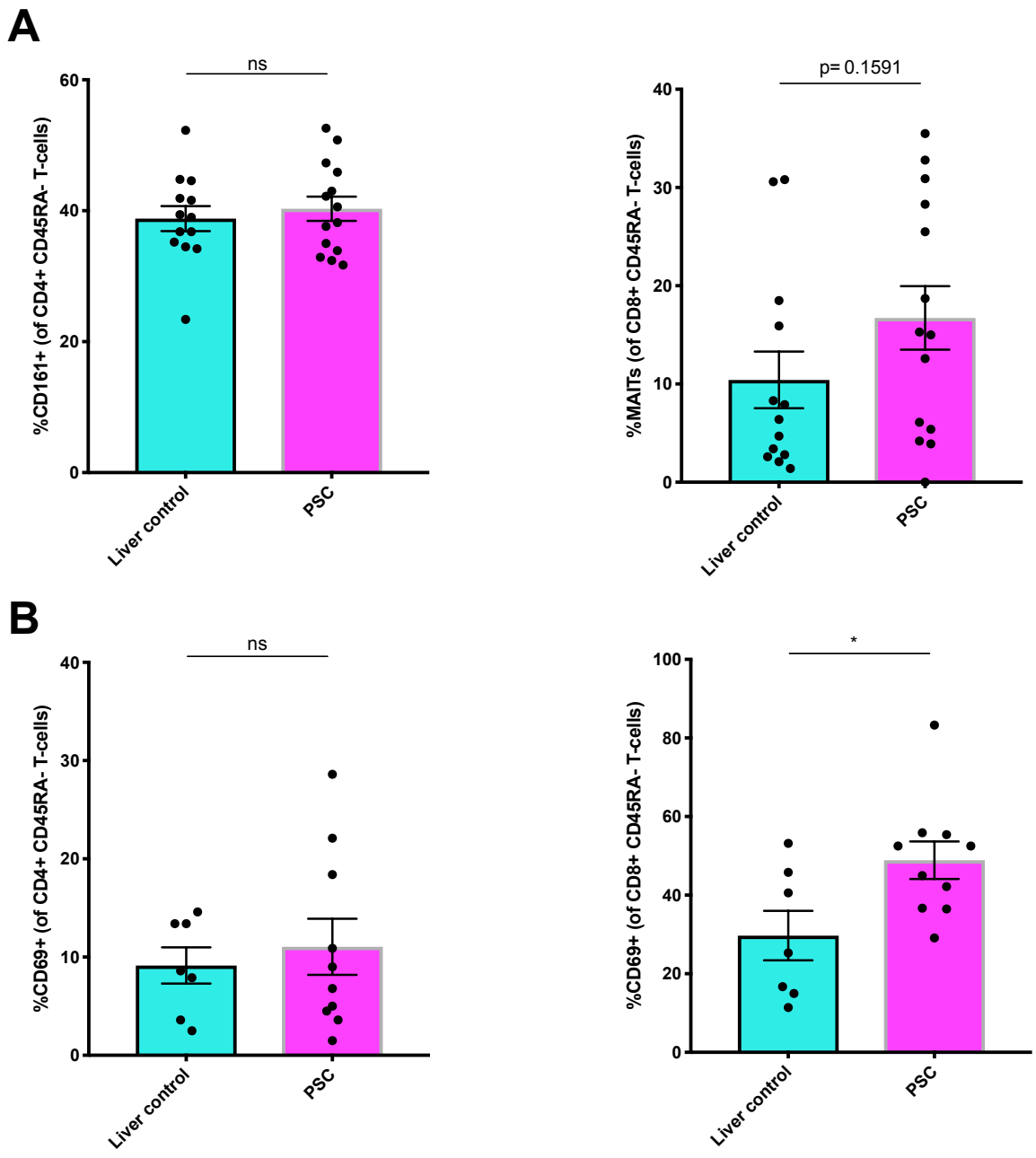


Figure 4-13 - CD161 and CD69 expression on liver-derived T-cells in PSC and controls.

LILs were isolated via FNA from patients with PSC and PC. A, the proportion of CD4+ memory T-cells which were CD161+ (left) and the proportion of CD8+ memory T-cells which were MAIT cells (CD161++ Vα7.2+, right) were compared between PSC and LC. There was a trend towards a higher proportion of MAITs in patients with PSC compared with LC. B, the proportion of CD4 memory T-cells (left) and CD8+ memory T-cells (right) which were CD69+ were compared between patients with PSC and LC. There was a higher proportion of CD8+ T-cells expressing CD69 among patients with PSC compared with LC. Graphs depict mean±SEM; unpaired t-test applied. FNA, fine needle aspirate; LC, liver controls; LIL, liver infiltrating lymphocytes; MAIT, mucosa associated innate-like T-cells; PSC, primary sclerosing cholangitis. *=p<0.05, ns=not significant, i.e. p>0.5.

4.4.5 Single cell sequencing of liver-derived lymphocytes and PBMC

For a subset of patients, leukocytes from liver FNAs and paired PBMC underwent FACS and single cell sequencing. Sorted cells were gated on singlets, alive, CD45+, and the lymphoid/myeloid population as per *Figure 4-14*. These CD45+ lymphoid/myeloid populations then underwent library preparation on the 10X Genomics platform and single cell RNA sequencing. Assistance with the FNA procedure was provided by radiologist, Dr Andrew Slater for these particular samples. Flow assisted cell sorting (FACS) was performed by Dr Helen Ferry within the flow cytometry facility at the Nuffield Department of Medicine, University of Oxford. Library preparation and RNA sequencing was carried out by colleagues at the Wellcome Centre for Human Genetics (WCHG) at the University of Oxford, Dr Hing-Yuen Yeung, Dr Moustaffa Attar, and Dr Hubert Swalinski. Bioinformatic analysis was carried out by bioinformatician, Dr Fabiola Curion, and assistance with subsequent analysis of the data output was given by Dr Calli Dendrou, both of whom are based at the WCHG.

4.4.5.1 Baseline demographics of patients in single cell sequencing project

Liver FNA and PBMC were successfully sorted from 13 patients (5 PSC, 6 NASH/NAFLD, 1 ALD, 1 HC [liver tissue adjacent to CRC metastasis]). However, scRNAseq sequencing data from the first 4 samples was not adequate enough for analysis. There was evidence of poor cell viability and also poor cell counts, and so these samples were not used. The resulting analyses were performed on a subset of 9 patients (3 PSC, 6 LC, the latter comprising of patients with NASH/NAFLD). The baseline demographics are shown in *Table 4.2.*

Table 4.2 - Baseline demographics of patients undergoing liver FNA for single cell RNA sequencing

Study ID	Liver diagnosis	Age (years)	Gender	Brunt fibrosis stage* (NAFLD/NASH only)	LSM (kPa)	INR	Platelets ($x10^9$/L)	ALP (IU/L)	ALT (IU/L)	Bilirubin (umol/L)	albumin (g/L)	Hb (g/L)
24	NASH	52.0	F	4	17.1	1	200	88	42	15	40	134
29	NASH	26.7	F	3	11.4	1	353	67	52	13	36	134
32	NASH	34.3	M	2	5.4	1	240	127	118	8	45	156
34	NASH	53.2	F	1	4.6	0.9	302	87	27	7	41	140
36	NAFLD	37.0	M	1	9.0	0.9	221	49	100	8	42	141
38	NASH	57.5	M	3	15.4	1	196	81	62	10	42	172
22	PSC/UC	54.2	M	n/a	12.3	1	378	552	162	55	30	126
27	PSC/UC	39.5	M	n/a	18.1	1	314	352	70	38	35	140
35	PSC/UC	45.4	M	n/a	n/a		313	382	134	23	44	143

*Brunt fibrosis stage is a component of the Brunt scoring system for NASH. The Brunt fibrosis stage is marked from 1 to 4, where 1 represents minimal fibrosis, and 4 is advanced fibrosis, or cirrhosis.[201] ALP, alkaline phosphatase; ALT, alanine transaminase; F, female; FNA, fine needle aspirate; Hb, haemoglobin; INR, international normalised ratio; LSM, liver stiffness measurement; M, male; n/a; not available; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PSC, primary sclerosing cholangitis; UC, ulcerative colitis.

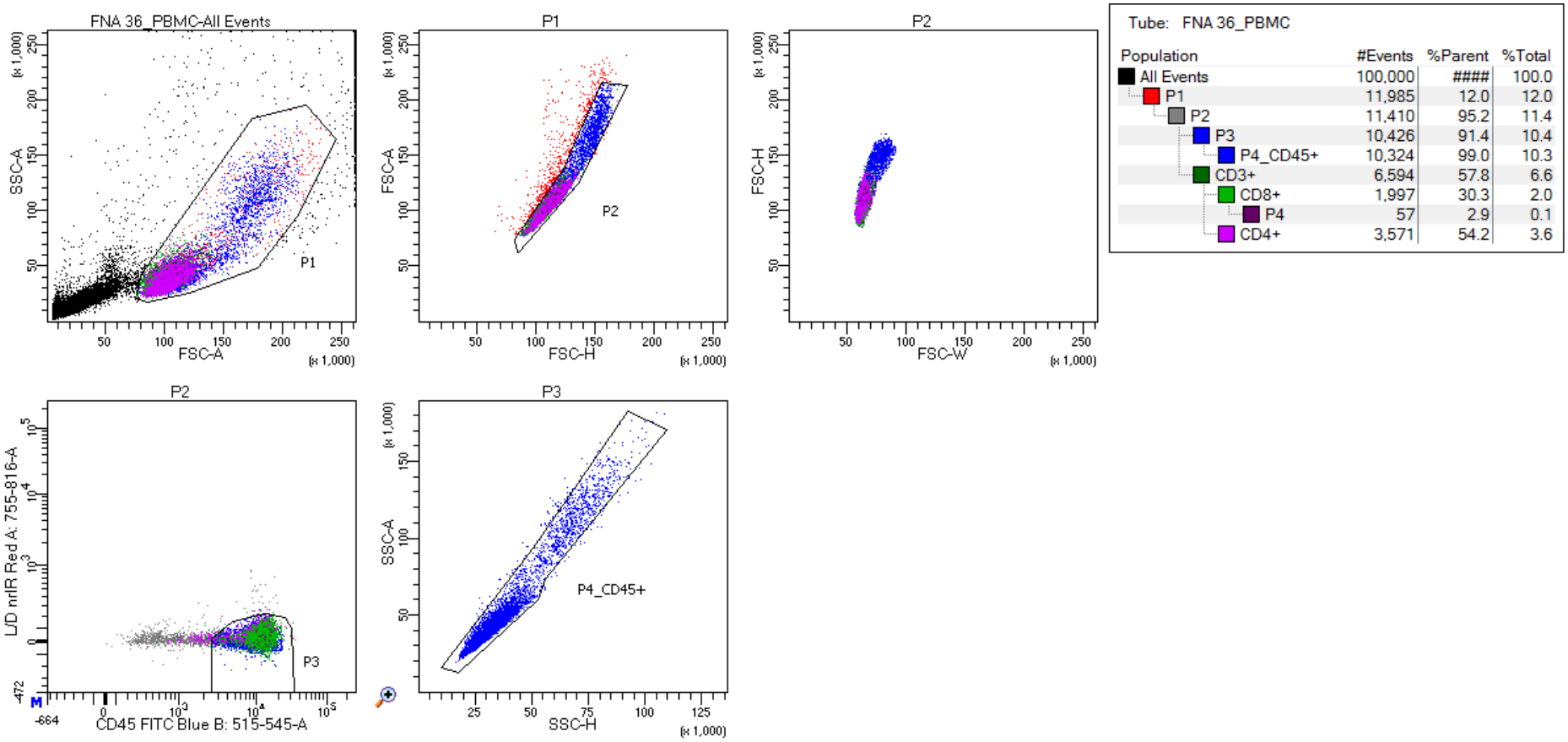


Figure 4-14 - Representative scatter plots showing gating strategy for FACS sorting of FNAs and PBMC for sequencing

Cells from liver FNA and paired PBMC were stained with cell surface markers then underwent FACS. The gating strategy went in the following order: P1 (lymphoid/myeloid population) > PS (singlets) > P3 (alive CD45+ cells) > P4 (further exclusion of doublets). The resulting sorted CD45+ lymphoid/myeloid cell population was then sent for RNA library preparation and single cell sequencing. These scatter plots are representative of a liver FNA sample. A, area; FACS, fluorescence-activated cell sorting; FNA, fine needle aspirate; FSC, forward scatter; H, height; PBMC, peripheral blood mononuclear cells; SSC, side scatter; W, width.

4.4.5.2 Quality of samples for sequencing

A selection of the quality control analyses are shown in *Appendix **8.6***. A reasonable sequencing saturation of both FNA and PBMC samples was obtained at roughly 80% across the board. The cell count for sequencing of FNAs was a mean 3907 cells (±SEM 690) and for PBMC was a mean 8183 cells (±1104). The median reads/cell for FNA samples ranged from 743 to 1012 (mean of medians was 881), and for PBMC samples, ranged from 742 to 1063 (mean of medians was 899).

4.4.5.3 Clusters identified among FNA and PBMC samples

t-SNE was applied to the FNA and PBMC samples at a resolution of 0.2 and seven clusters were identified (see *Figure 4-15*). Each cluster resembled specific leukocyte subsets as analysed by the main gene reads for each cluster (see *Figure 4-16*). Importantly, there was a distinct cluster which was only present among FNA samples, and not among PBMC samples, indicating a unique liver-specific subset derived from FNA (cluster 6, pink).

Clusters 0 (red) and 1 (yellow) were clearly T-cells as denoted by their high expression of T-cell receptor (TCR) genes CD3E and CD3G. CD4 is traditionally not detected very well on RNA sequencing but cluster 1 was shown to be a CD8+ T-cell cluster by its high expression of CD8A and CD8B, as well as various genes for cytotoxic granules such as multiple granzymes. Cluster 0, in addition to expressing genes for TCR, expressed many chemokine receptors, and was denoted as a CD4+ cluster. Cluster 2 (green) expressed many NK cell markers such as Natural Killer Granule Protein 7 (NKG7), various perforin genes (e.g. PRF1), killer cell receptors and granzymes. The other smaller clusters were categorised as the most likely cell types in a similar fashion by analysing the genes most highly expressed by each individual cluster.

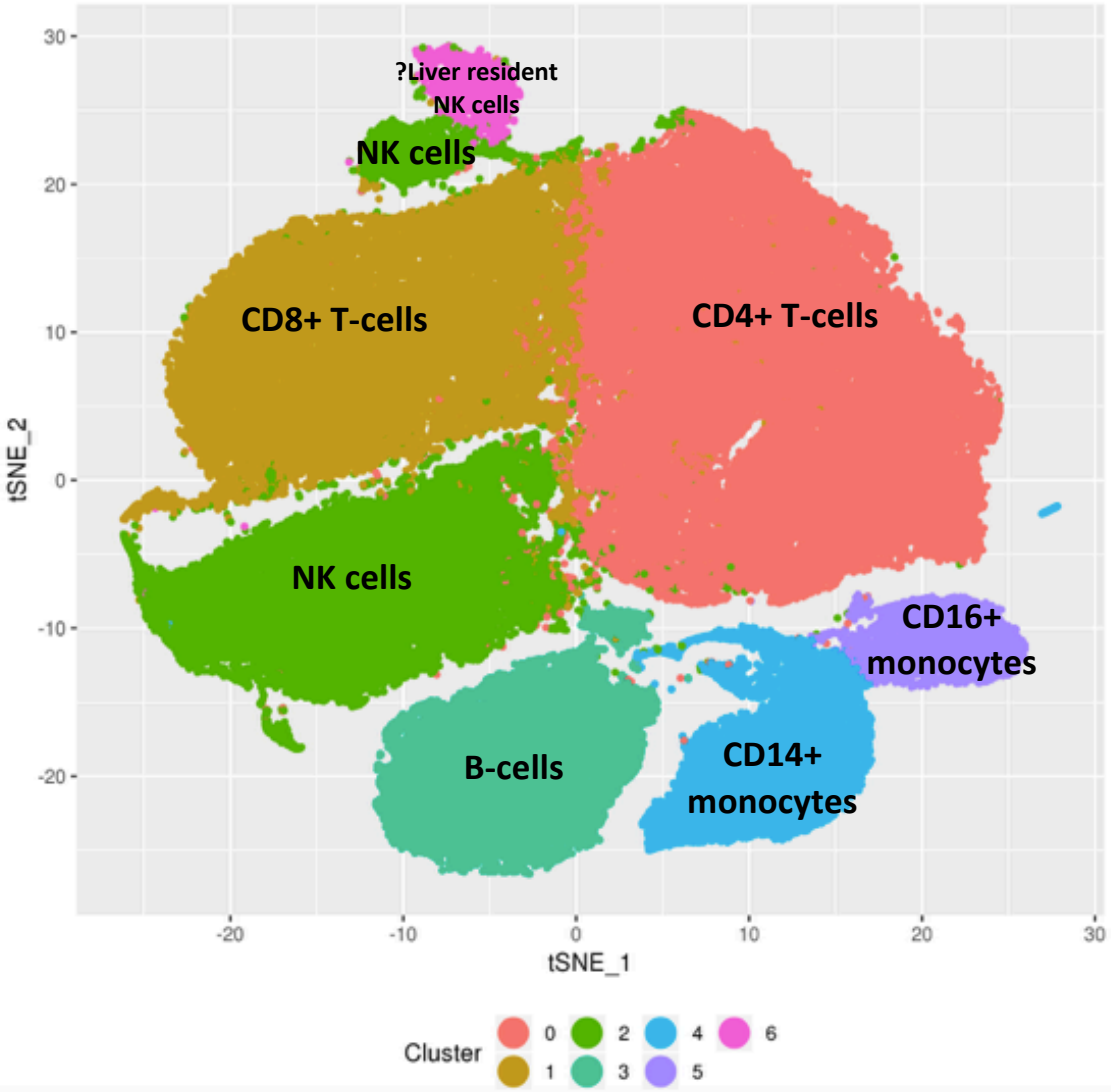
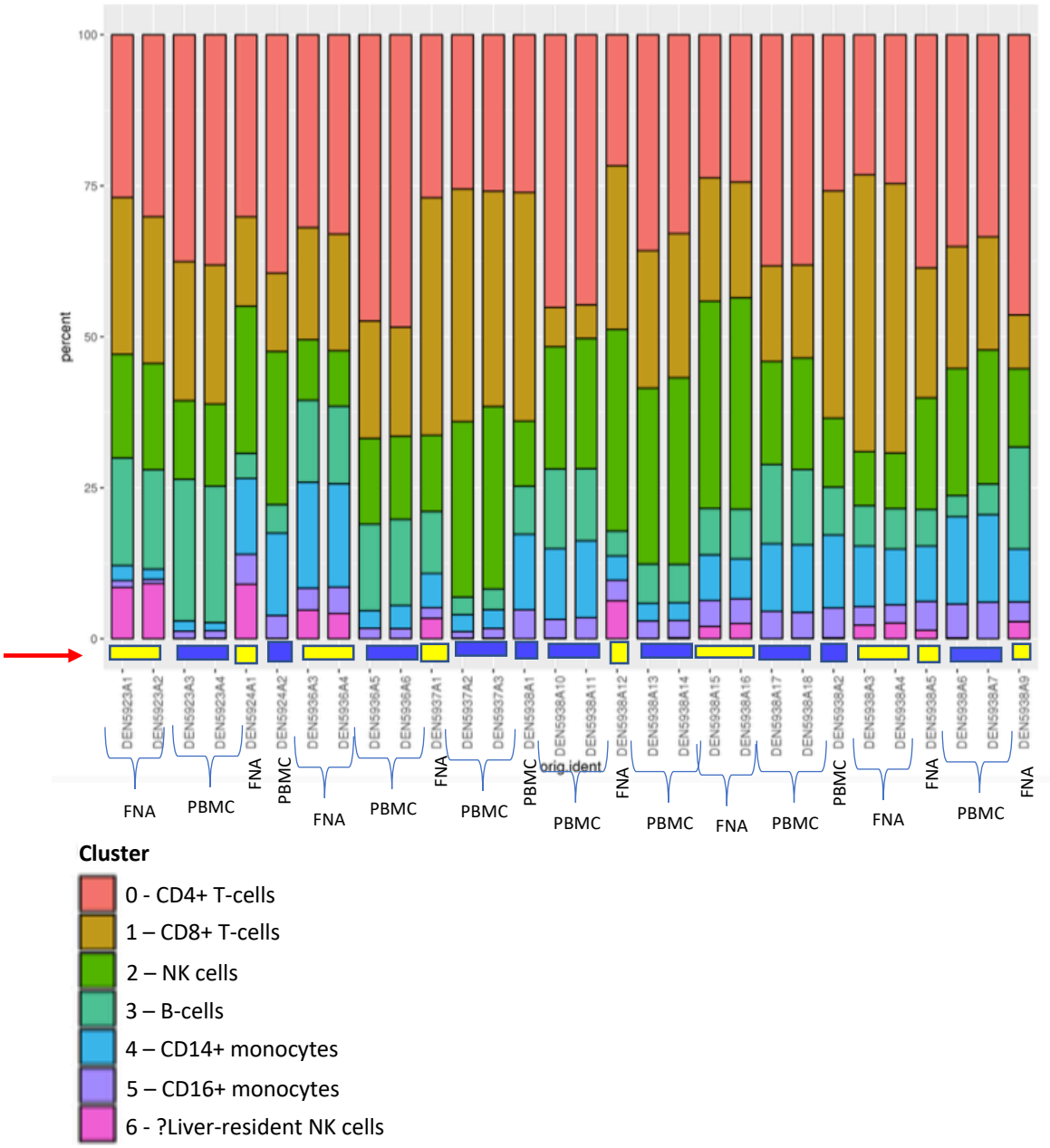


Figure 4-15 - t-SNE of combined sequencing data from FNA and PBMC samples demonstrates 7 unique clusters

The transcript data from sequencing of 9 liver FNAs and 9 PBMC (sorted CD45+ lymphoid/myeloid populations) was pooled and t-SNE was applied at a resolution of 0.2. Seven distinct clusters were identified according to differential gene expression. The likely identification of the various clusters is depicted according to the highest expressed genes for each cluster. FNA, fine needle aspirate; NK, natural killer; PBMC, peripheral blood mononuclear cells; t-SNE, t-distributed stochastic neighbour embedding.


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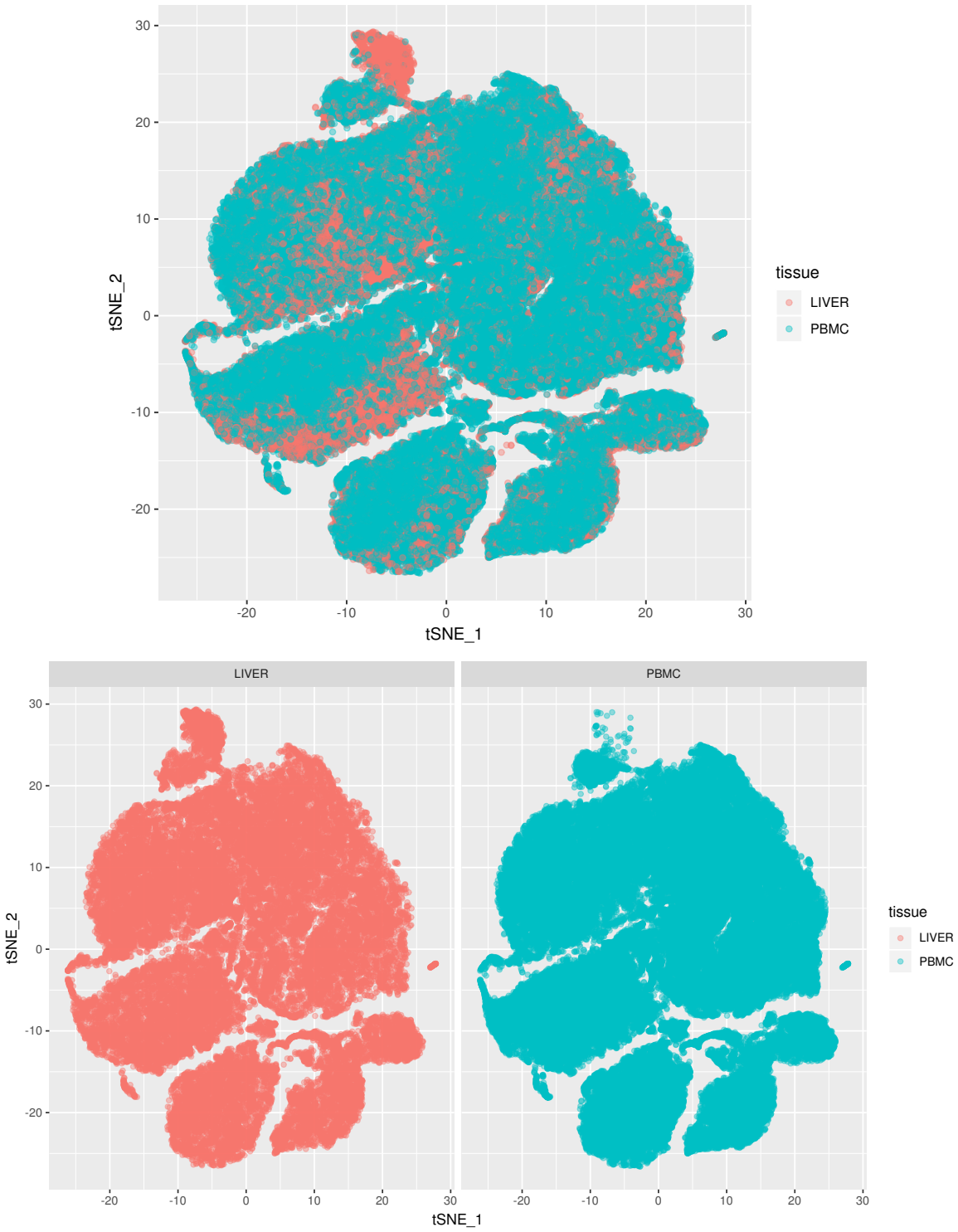


Figure 4-17 – t-SNE plots comparing transcriptional clusters derived from liver versus peripheral blood samples.

t-SNE analysis of liver (red) and peripheral blood (blue) derived samples. Cluster 6, as denoted in *Figure 4-15* is only present in the liver t-SNE as compared with the PBMC t-SNE. The top graph shows the two types of samples overlaid, and the bottom graphs show liver- and peripheral blood-derived samples separately. FNA, fine needle aspirate; PBMC, peripheral blood mononuclear cells; T-SNE, t-distributed stochastic neighbour embedding.

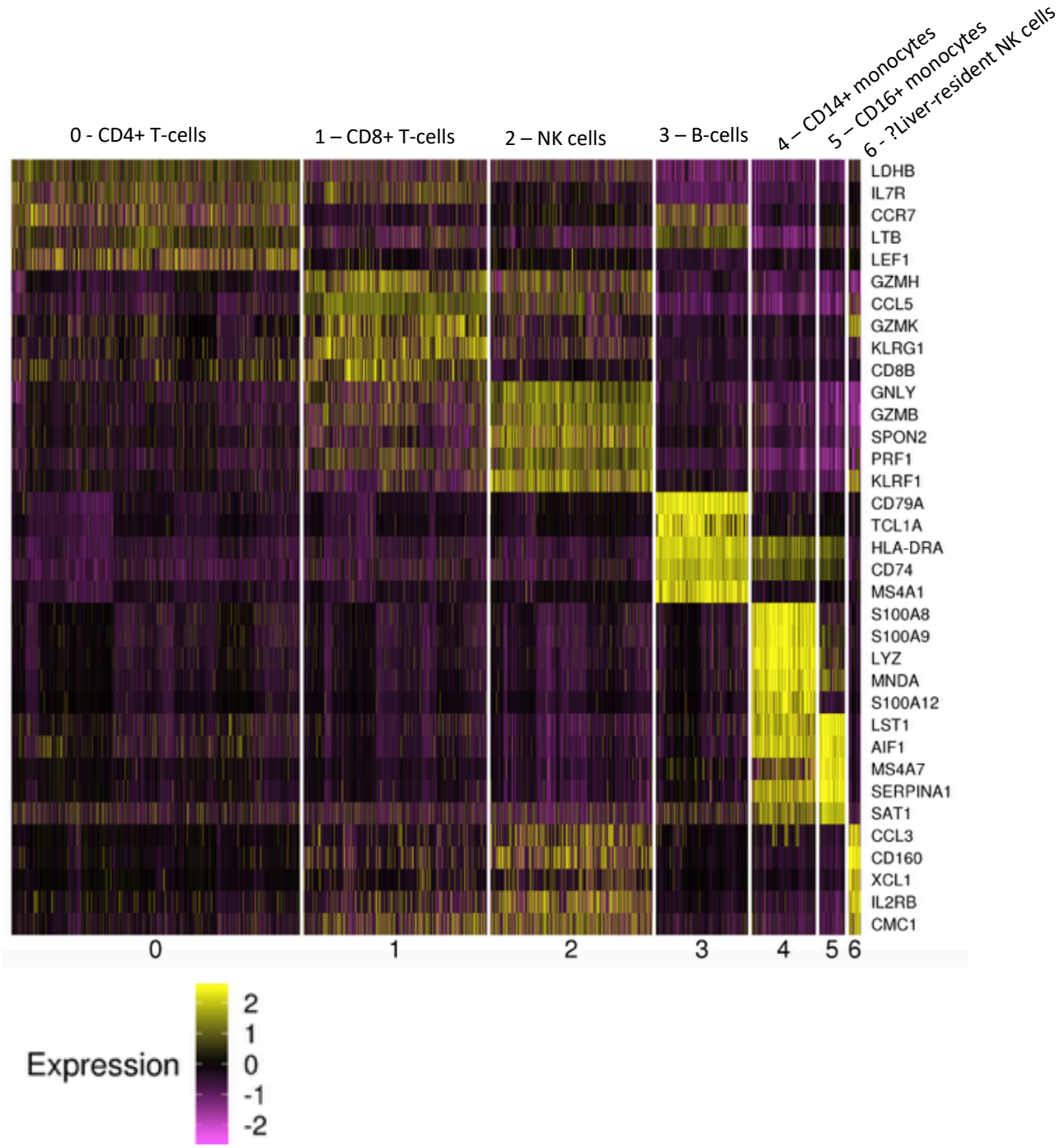


Figure 4-18 - Heat map demonstrating the top 5 genes expressed for each cluster among FNA and PBMC samples

Combined t-SNE of PBMC and FNA liver revealed 6 distinct clusters, and this heat map displayed the top 5 differentially expressed genes for each cluster based on the average log fold change. FNA, fine needle aspirate; PBMC, peripheral blood mononuclear cells; T-SNE, t-distributed stochastic neighbour embedding.

4.4.5.4 Gene expression within individual clusters

The gene expressions within each cluster are shown in *Figure 4-19* and *Figure 4-20.* There was very little CCR9 expression detected from sequencing and transcripts that were detected were mainly on CD4+ and B-cells. Integrin β7 (ITGB7) was expressed mainly on CD4+ T-cells, CD8+ T-cells, B-cells and NK-cells, whereas Integrin α4 (ITGA4) was fairly ubiquitously expressed throughout clusters which may represent some expression of α4β1.

There was higher CD69 expression on liver-derived samples compared with PBMC, with a predominance on CD8+ T-cells and NK cells, consistent with the flow cytometry data. Interestingly, lymphocyte activating gene-3 (LAG-3), an activation and exhaustion marker on T-cells, was detected, mainly on CD8+ T-cells, and more so in peripheral blood compared with liver. The meaning of this is unclear.

KLRB1, the gene for CD161, was expressed, as expected, on CD8+ T-cell and NK cells strongly, as well as some CD4+ T-cell expression. However, it was more intensely expressed in the liver compared with PBMC samples, consistent with the findings from flow cytometry of greater MAITs and NK cells in the liver FNAs compared with peripheral blood. Finally, FCER1-A, the gene for Immunoglobulin E receptor, was strongly expressed on a tail portion of cluster 4 which was identified as a CD14+ monocyte cluster. This may actually represent another cell phenotype, such as basophils or dendritic cells, for which the resolution of 0.2 for the t-SNE was too low to characterise.

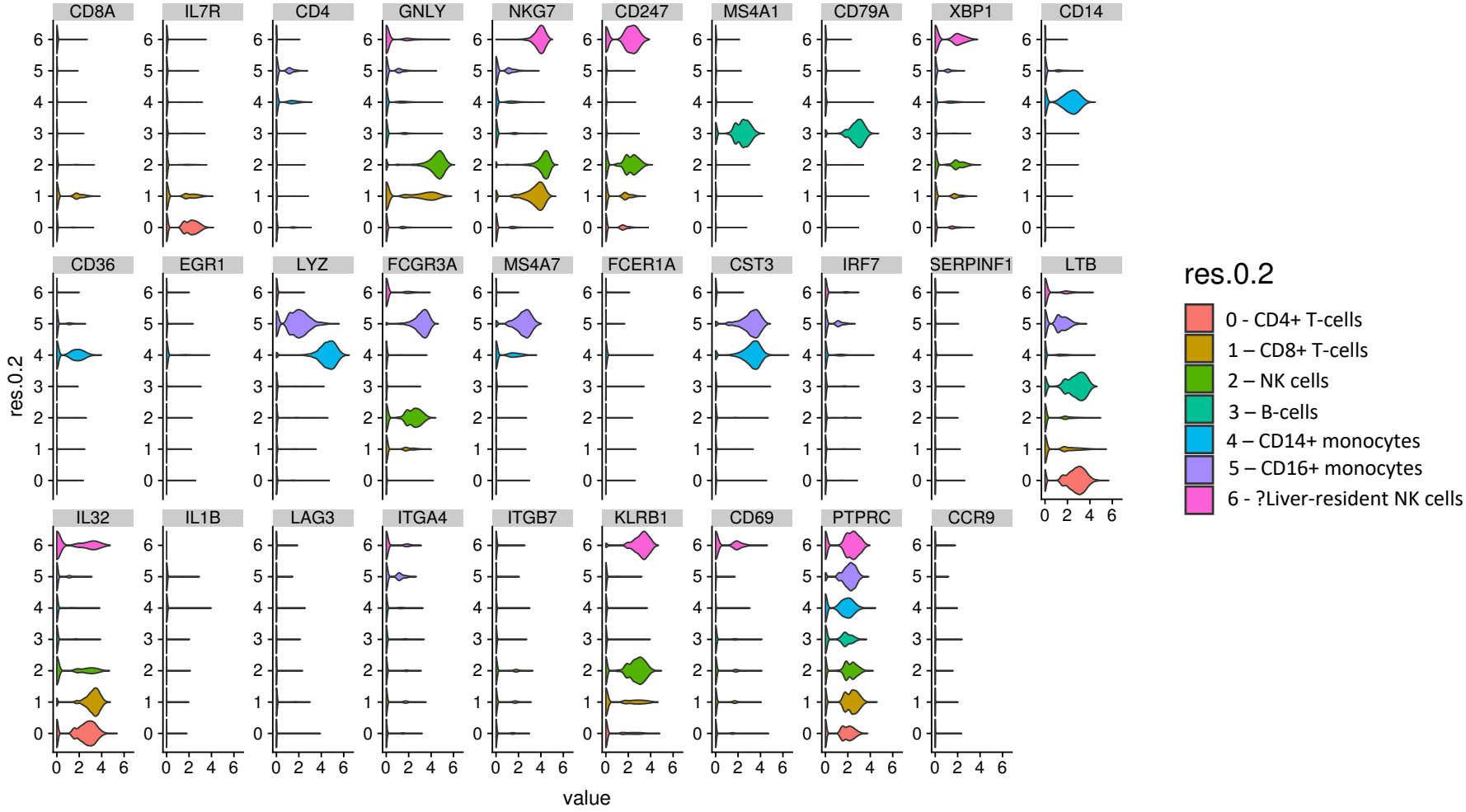


Figure 4-19 - Violin plots showing marker gene expressions among the 6 clusters (PBMC and liver FNAs combined)

For each marker expression probability distributions across clusters are shown. Clusters were derived using a resolution of 0.2; Res, resolution.

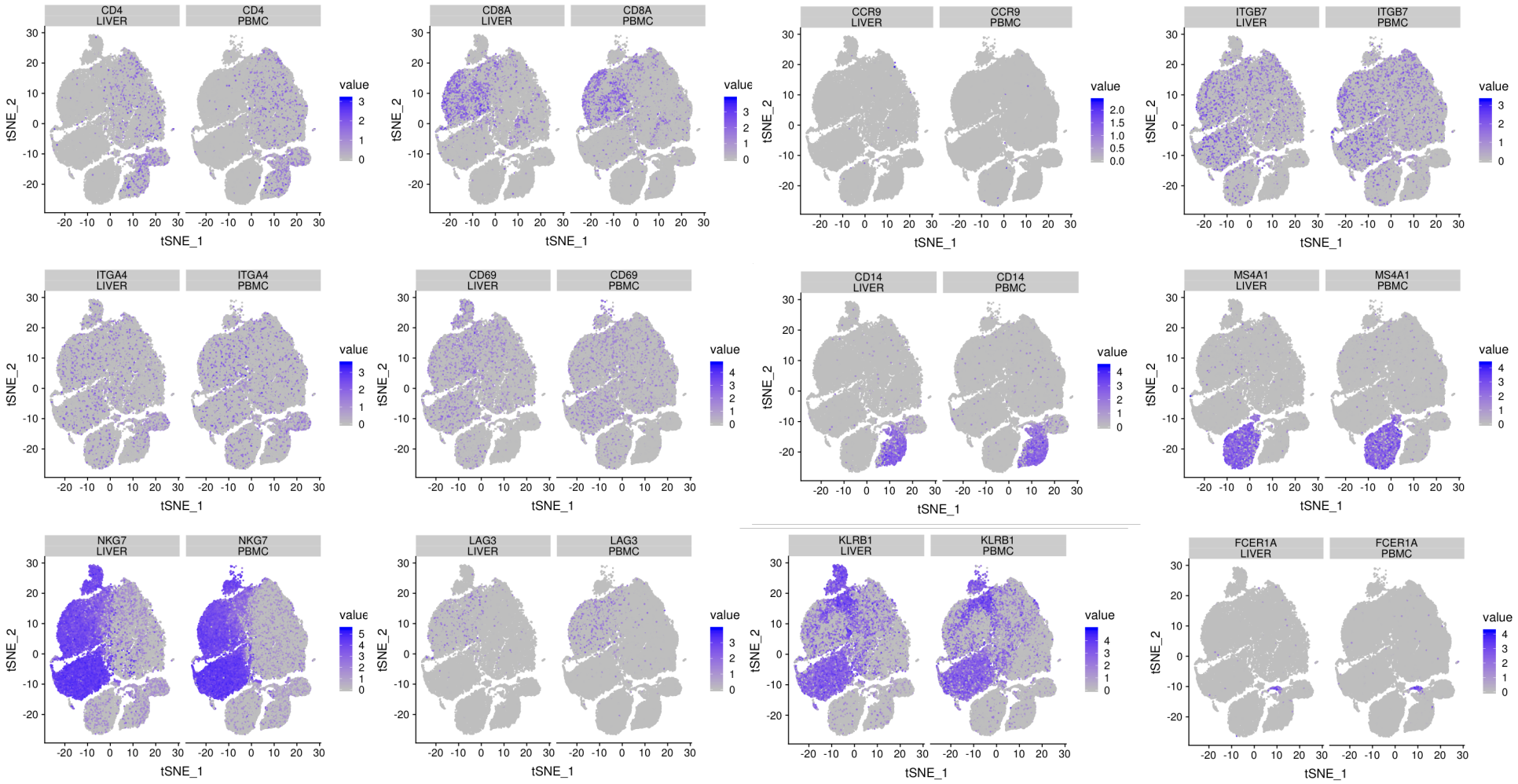


Figure 4-20 - t-SNE plots showing various marker gene expressions within liver FNA samples and PBMC samples.

4.4.5.5 Gene expression comparisons between PSC and liver controls

t-SNE plots were generated for FNA liver samples and PBMC samples comparing PSC with LC. Particularly among the CD4+ cluster and to a lesser extent the B-cell cluster, there appeared to be differentiation between the two conditions within the liver (see Figure 4-21). These differences have not been fully delineated. It was felt that a resolution of 0.2 was too low to properly ascertain the many different clusters/ phenotypes of cells which exist within the liver, and therefore, the aim is to go back with the single cell bioinformaticians and re-analyse the data at a higher resolution and from there tease out the differential gene expression between PSC and LC.

In addition, alternative data analysis approaches, including separate clustering of PSC and LC liver and PBMC data followed by meta-analysis (as opposed to initial clustering of all data), as well as employment of the Uniform Manifold Approximation and Projection (UMAP) algorithm, are currently being tested. UMAP is a non-linear dimensionality reduction technique that has recently been shown to have superior consistency and more meaningful cell cluster organisation and preservation of continuums compared to t-SNE when analysing high-dimensional flow cytometry and scRNAseq data.[202] Regardless, it should be noted that it is unlikely that CCR9 will be borne out as a differentiated gene between PSC and LC from these data given the low expression of CCR9 transcript when analysing the whole cohort.

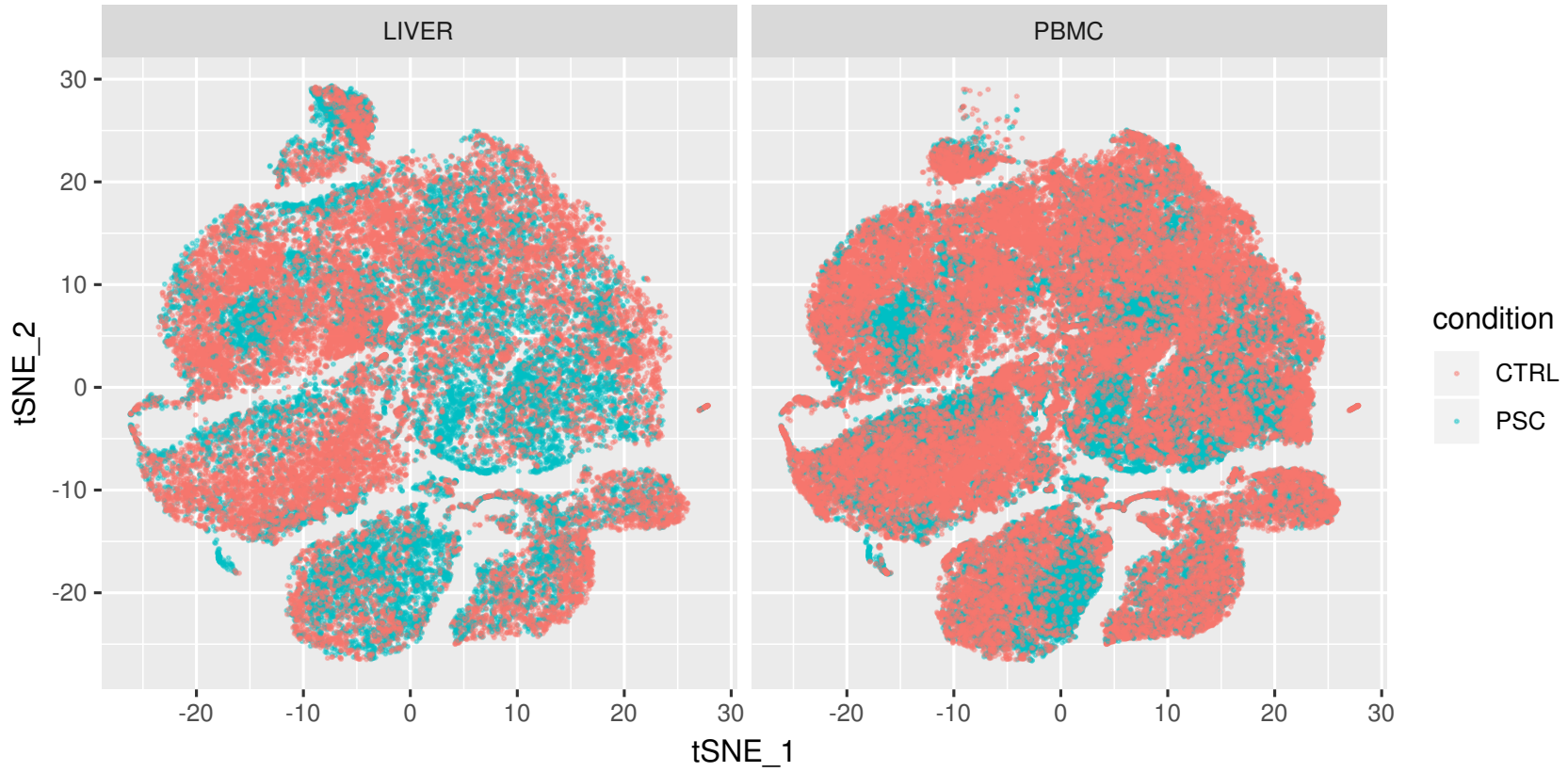


Figure 4-21 - t-SNE plots of liver- and peripheral blood-derived samples showing differential gene expression between PSC and liver controls.

t-SNE was applied to gene expression data from single cell-RNA sequencing on lymphoid/myeloid populations derived from liver (FNA) and peripheral blood. Samples were obtained from patients with PSC (blue, n=3) and liver controls (red, n=6). The clusters depicted are the same as those shown in *Figure 4-15*. FNA, fine needle aspirate; PBMC, peripheral blood mononuclear cells; t-SNE, t-distributed stochastic neighbour embedding.

4.4.6 CD3 and CCR9 expression as seen on liver IHC in patients with PSC and controls

4.4.6.1 Baseline demographics

IHC was performed on 36 FFPE liver biopsies (10 PSC/IBD, 12 PSC/no IBD, 14 LC). The baseline demographics of these patients is shown in *Table 4.3.* Patients with PSC were split into two groups (PSC/IBD and PSC/no IBD) in case the presence of IBD affected the presence of gut-homing lymphocytes within the liver. Patients with PSC/IBD were younger than LC, and numerically younger than those with PSC/no IBD (latter comparison not statistically significant). Similarly, there was a trend towards patients with PSC/IBD having a higher median ALP than LC, though the histological fibrosis and activities scores were not different among the three groups.

4.4.6.2 Quality and location of staining for CD3 and CCR9

CD3 staining was intense on both cell membranes and there was some cytoplasmic staining of mononuclear cells (see Figure 4-22). CCR9 staining was not as intense as CD3 staining, nor as dark as staining of CCR9+ intestinal samples (see Figure 4-23). There was some minor background staining of hepatocyte cytoplasm in some biopsies when working up the protocol, and so a more dilute concentration was used for CCR9 staining within the liver compared with the intestine to allow clear differentiation between CCR9+ lymphocytes and background staining. However, as a result, CCR9 staining of lymphocytes was fairly faint, but nevertheless still present so one could count positive cells.

Table 4.3 - Baseline demographics of patients whose liver biopsies were analysed by immunohistochemistry

Variable	PSC/IBD (n=10)	PSC/ no IBD (n=12)	LC (n=14)	p-value*
Age, years, mean (±SEM)	33 (±4.4)*	41 (±3.6)	51 (±3.2)*	0.006
Male gender, n (%)	7 (70.0%)	5 (41.7%)	4 (28.6%)	0.107
Type of liver disease, n (%)				
PSC	8 (80%)	11 (91.7%)	n/a	n/a
PSC/AIH overlap	2 (20%)	1 (8.3%)	n/a	
Small duct-PSC	0 (0%)	0 (0%)	n/a	
PBC	n/a	n/a	8 (57.1%)	n/a
AIH	n/a	n/a	3 (21.4%)	
NASH/NAFLD	n/a	n/a	3 (21.4%)	
ALD	n/a	n/a	1 (7.1%)	
Type of IBD, n (%)				
Ulcerative colitis	7 (70%)	n/a	n/a	n/a
Crohn's disease	2 (20%)	n/a	n/a	
IBD-unspecified	1 (10%)	n/a	n/a	
Previous IBD surgery[#], n (%)	5 (50%)	n/a	n/a	n/a
Baseline liver biochemistry				
ALP, median (IQR)	360 IU/L* (179-710)	188 IU/L (83-544)	93 IU/L* (67-239)	0.0647
ALT, median (IQR)	91 IU/L (50-290)	102 IU/L (33-252)	75 IU/L (44-156)	0.843
Histology^, median (IQR)				
Ishak fibrosis score	3.0 (2.0-5.0)	2.0 (1.3-4.5)	3.0 (2.0-5.5)	0.612
Ishak activity score	3.0 (2.5-5.0)	3.5 (2.0-6.0)	5.0 (3.5-7.0)	0.321

*Ordinary one-way ANOVA or Kruskal-Wallis test performed, depending on distribution of values; a further * denotes the individual values to which any significant or near-significant p-values refer calculated by Tukey's or Dunn's multiple comparisons test. The one exception was for gender where Chi-square test was performed. [#]All surgeries consisted of a pan- or subtotal colectomy; 3 patients had ileal pouch anal anastomoses, 1 patient had an ileostomy, and 1 patient had a ileorectal anastomosis. ^The histological scoring system used was the Ishak system,[203] and the fibrosis component (score out of 6) and activity score (score out of 18) is shown. AIH, autoimmune hepatitis; ALD, alcoholic liver disease; ALP, alkaline phosphatase; ALT, alanine transaminase; IBD, inflammatory bowel disease; LC, liver controls; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

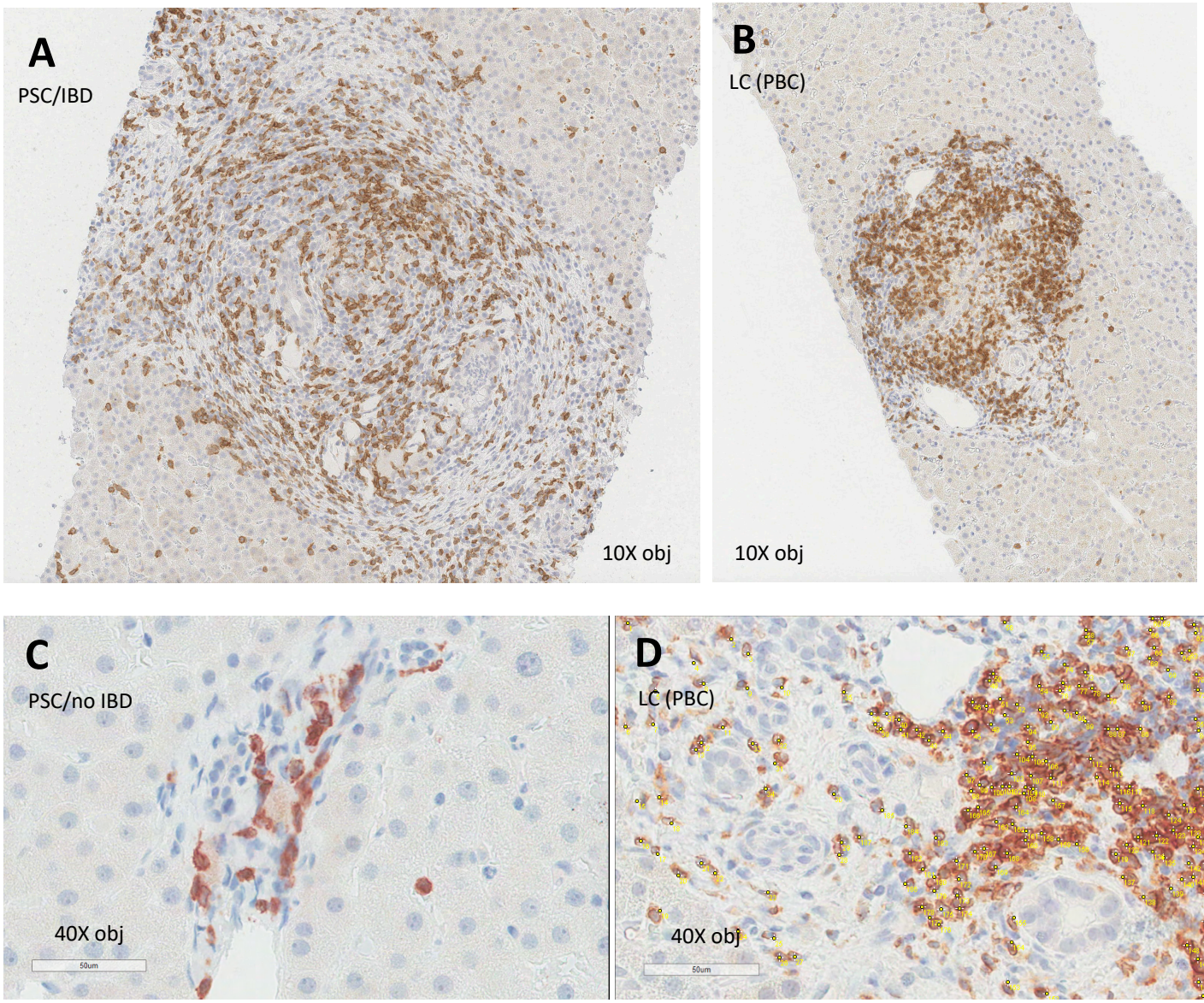


Figure 4-22 - Immunohistochemical staining of liver biopsies for CD3

Representative images of liver biopsies stained with CD3 antibody (brown) and counterstained with Haematoxylin (blue). A, Low power image centred on a portal tract of a patient with PSC/IBD showing numerous CD3+ T-cells around the portal tracts with a characteristic onion skin fibrosing appearance of PSC. B, Low-power image of a liver biopsy from a patient with PBC, again showing numerous CD3+ T-cells within the portal tracts. C, HPF of a portal tract of a patient with PSC/ no IBD with only a few CD3+ T-cells. D, HPF of a patient with PBC (LC) with numerous CD3+ T-cells within the portal tract and representative counting of CD3+ T-cells using the counting tool in Image J. A&B are at 10X objective (i.e. 100X magnification) and C&D are at 40X objective (i.e. 400X magnification) and are at the magnification used for definition of HPF. LC, liver control; HPF, high power field; IBD, inflammatory bowel disease; obj, objective; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

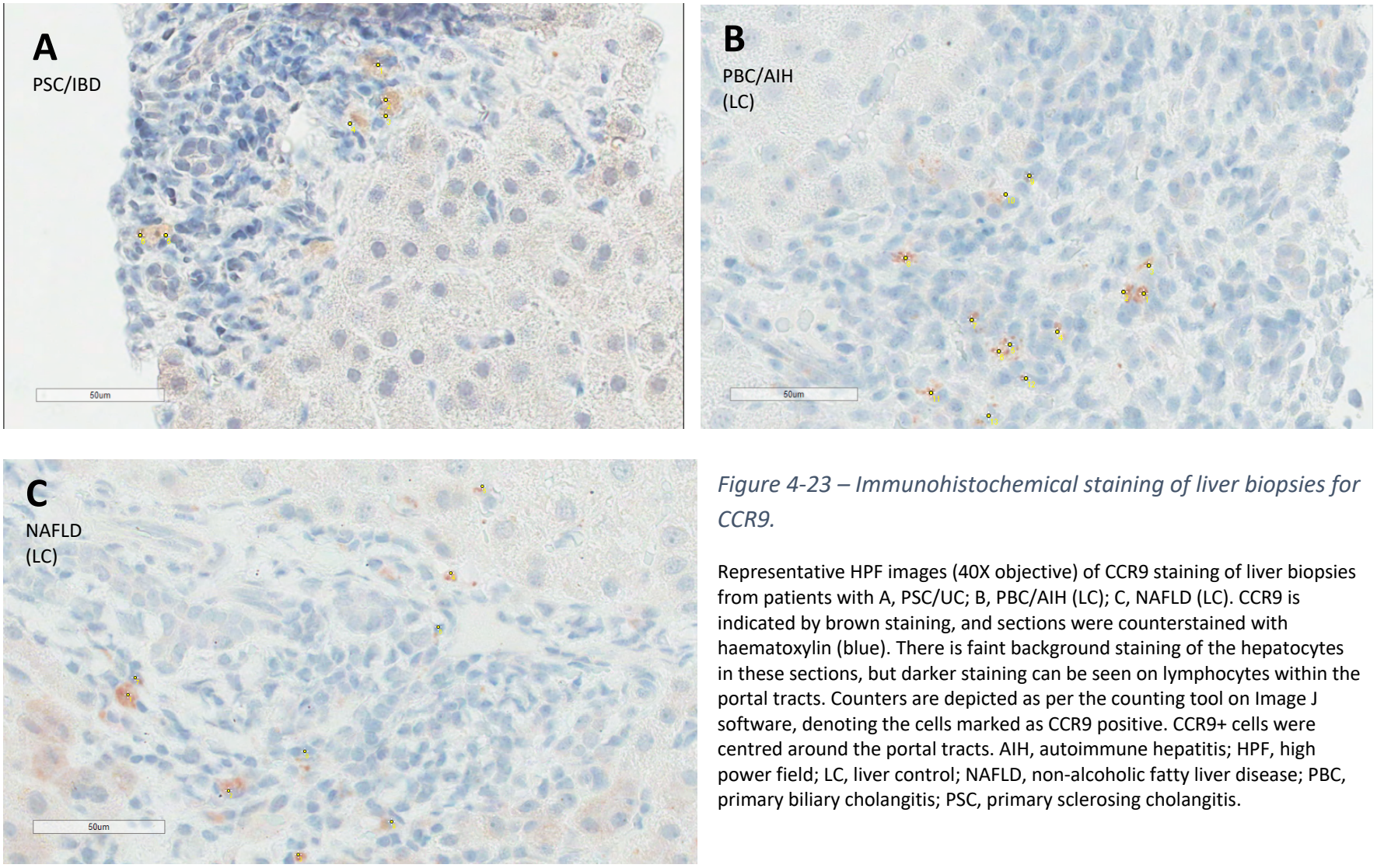


Figure 4-23 – Immunohistochemical staining of liver biopsies for CCR9.

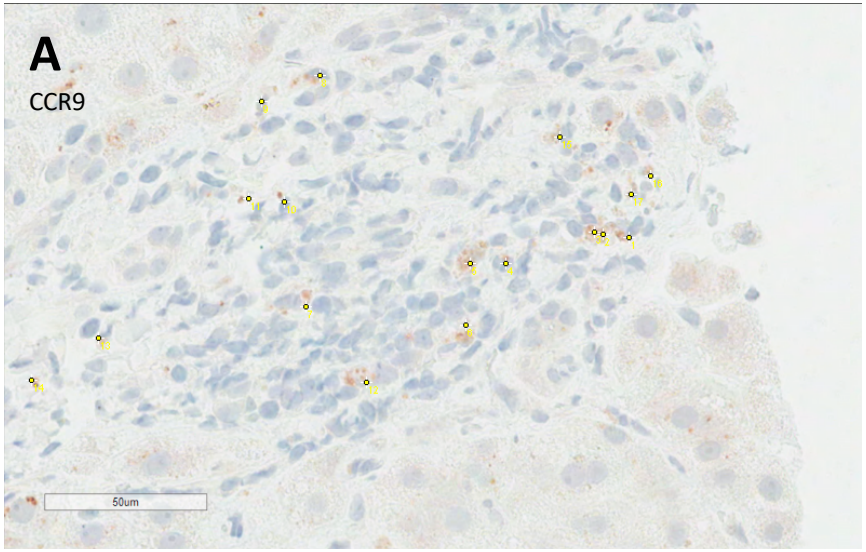
Representative HPF images (40X objective) of CCR9 staining of liver biopsies from patients with A, PSC/UC; B, PBC/AIH (LC); C, NAFLD (LC). CCR9 is indicated by brown staining, and sections were counterstained with haematoxylin (blue). There is faint background staining of the hepatocytes in these sections, but darker staining can be seen on lymphocytes within the portal tracts. Counters are depicted as per the counting tool on Image J software, denoting the cells marked as CCR9 positive. CCR9+ cells were centred around the portal tracts. AIH, autoimmune hepatitis; HPF, high power field; LC, liver control; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

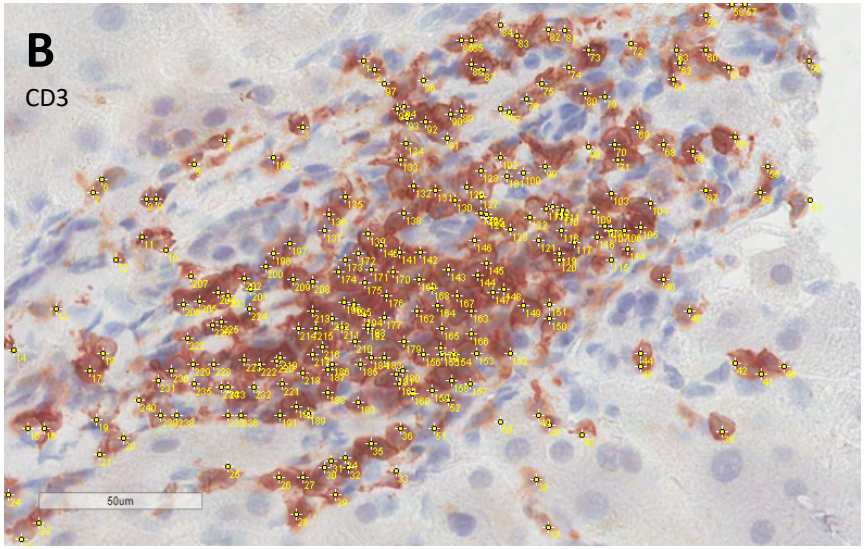
In some cases, punctate brown pigment/ staining could be seen within the cytoplasm of hepatocytes of unclear significance (see Figure 4-24). This did not occur in the negative controls done during the same experiment, so it seemed to be specific to the CCR9 antibody.

In all experiments, the negative controls were negative, and the positive control for CCR9 (either an ileal or colonic section) were always positive, indicating that the experiment had worked correctly each time.

CCR9 staining was successful on all 36 biopsies, and CD3 staining was available for 33 of 36 sections. In 3, CD3 staining data was not available due to one staining completely negative (despite positive controls working, and CCR9 staining working for the same biopsy – felt to be false negative), and in two there were issues with the scanned image rendering them inadequate for counting.

CCR9+ lymphocytes were centred around the portal tracts, as were the CD3+ T-cells. The distribution of the mean counts/5HPF were non-parametric for both CD3 and CCR9, so the median of the mean counts/5HPF is reported and compared among groups.





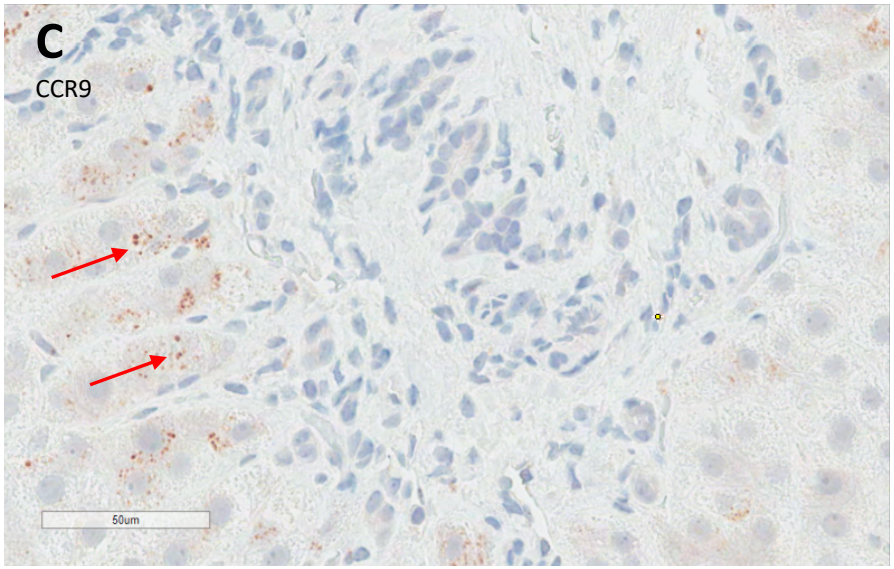


Figure 4-24 - Matched HPF for CCR9 and CD3, and aberrant CCR9 staining

A and B are matched HPF where A shows CCR9 staining and B shows CD3 staining. Both show the counters for the positive respective cells. Matched HPFs were used to calculate the CCR9/CD3 ratio. These sections are from a liver biopsy from a patient with AIH (LC). C, CCR9 staining caused some faint background hepatocyte cytoplasmic staining, but also occasionally some punctate brown staining within the hepatocytes (red arrows), which did not occur in the negative controls. It is not clear what this represents, but these cells were not counted, as only CCR9+ lymphocytes were counted. This section is from the same patient as in A&B (different HPF). Only one lymphocyte is CCR9+ on this HPF. All images are at 40X objective. AIH, autoimmune hepatitis; HPF, high power field; LC, liver control.

4.4.6.3 CCR9 and CD3 counts within the liver

There was no difference in the median count CD3/5HPF among the differing conditions, though there was a wide range of values: from 17.6/5HPF to 298.6/HPF. The median count CD3/5HPF for each condition was: PSC/IBD 86.5 (IQR 62.0-114.7); PSC/ no IBD 72.3 (IQR 48.4-115.3); LC 110.3 (IQR 57.0-176.3, p=0.447, see *Figure 4-25*).

There was a trend towards higher median count CCR9/5HPF in PSC/IBD (3.4 [IQR 0.2-5.2]) compared with PSC/no IBD (0.5 [IQR 0.1-3.0]) and LC (2.0 [IQR 0.6-6.0]) but it was not statistically significant (p=0.352). The distribution of the mean counts of CCR9/5HPF was interesting in that half (18/36) of the values were very low (<1/5HPF) and the other half ranged from 3-15. 8/36 (22.2%) had a mean count CCR9/5HPF>5: 2 PSC/IBD, 1 PSC/no IBD (who also had AIH overlap), and 4 LC (PBC, AIH cirrhosis, ALD cirrhosis, and PBC/AIH).

In case the CCR9 results was influenced by the number of CD3 cells present, a CCR9/CD3 ratio was calculated for each individual, and then these values were compared among conditions. As the sections stained for CCR9 and CD3 were adjacent to each other for each individual, morphologically it was possible to match the HPF, and it was therefore a true reflection of the proportion of CD3+ T-cells which were CCR9+ for each HPF.

Once again, there was a trend towards a higher median CCR9/CD3 ratio among patients with PSC/IBD (0.033 [IQR 0.001-0.838]) compared with PSC/no IBD (0.006[0.000-0.053] and LC (0.011 [IQR0.003-0.036], p=0.556). These values are equivalent to 3.3% vs. 0.6% vs. 1.1% respectively. However, as with the CCR9 counts, the CCR9/CD3 ratios were split into two groups – low to negligible values and high values of 4-11%.

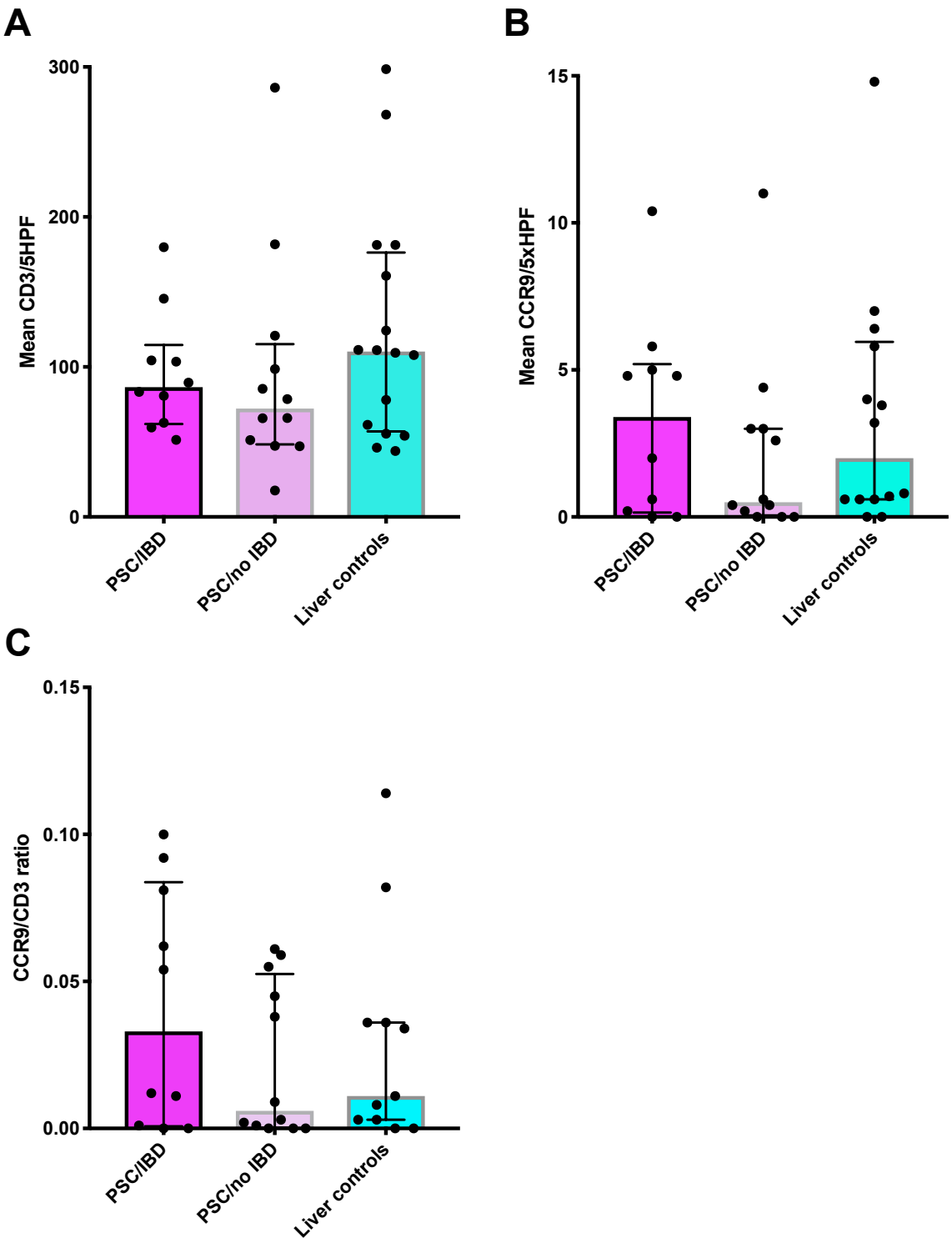


Figure 4-25 - Mean counts of CCR9 and CD3 on immunohistochemical analysis of the liver in patients with PSC/IBD, PSC/no IBD and liver controls.

Five HPF were selected on each liver biopsy which had the highest concentration of CCR9+ lymphocytes. The number of CCR9+ cells (A) and CD3+ cells (B) were calculated for each of the 5 HPFs and a mean count was determined. This mean count/HPF was compared among patients with PSC/IBD, PSC/ no IBD, and LC. C, the ratio of the mean CCR9/5HPF to mean CD3/5HPF was determined and compared among groups. Bars represent the median values with IQR shown. Kruskal-Wallis test showed there were no statistically significant differences among A, B, and C. LC, liver controls; HPF, high power field; IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis.

4.4.6.4 Correlation between CCR9 counts and various liver and demographic variables

The question was therefore raised as to whether there was any correlation of high CCR9 counts and/or CCR9/CD3 ratio and inflammatory activity or fibrosis.

Whilst there was a minor trend, there was no correlation found between CCR9 mean counts/5HPF and Ishak's fibrosis score (Pearson's r=0.299, p=0.086, see *Figure 4-26*). This was also true if one looked for a correlation within each disease group separately. Furthermore, there was no correlation with the Ishak activity score (Pearson's r=0.135, p=0.445) nor its sub-components: portal inflammatory score (marked out of 4, Pearson's r=0.296, p=0.089) and interface involvement score (marked out of 4, Pearson's r=-0.119, p=0.047). There was also no correlation with ALP or ALT. All these parameters were checked for a correlation with CCR9/CD3 ratio and none was found (data not shown).

Baseline demographics and CCR9 counts were then analysed for any association or correlation. There was no correlation of mean counts of CCR9/5HPF and age, however, interestingly, males had a higher mean count of CCR9/5HPF (4.7 ±SEM 1.1) compared with females (1.9 ±0.4, p=0.008, see *Figure 4-27*). Males also had a higher median CCR9/CD3 ratio compared with females. In case this was due to a predominance of males within the PSC/IBD group, males and females were compared within the PSC/IBD group alone. Males still had higher CCR9 count and CCR9/CD3 ratio numerically compared with females, though numbers were obviously small and therefore not statistically significant.

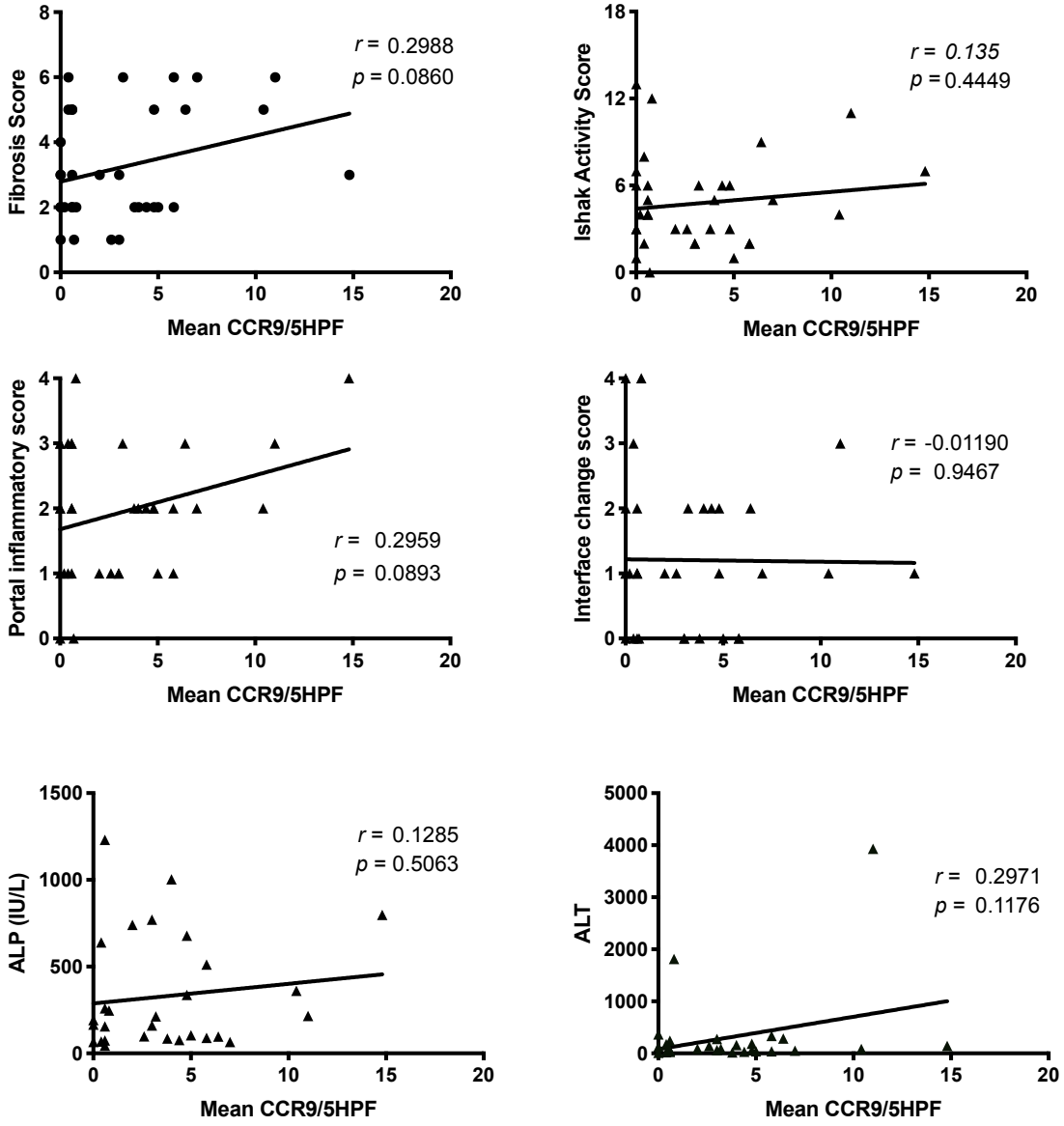


Figure 4-26 - The correlation between different baseline variables and the mean count of CCR9 positive cells per 5 high power fields.

The hepatic mean CCR9/5HPF was determined for each patient (n=36) as per *Figure 4-25*. This was plotted against various variables for each patient: Ishak fibrosis score, Ishak Activity score and two of its subcomponents (portal inflammatory score and interface change score) , ALP and ALT. A line of best fit via linear regression was plotted, and Pearson's co-efficient was calculated. There was a trend towards a weak correlation of mean CCR9/5HPF values with both fibrosis score and portal inflammatory score, but these were not statistically significant. Also, there appeared to be a trend towards a correlation between rising ALT and rising mean CCR9/5HPF, however there were two significant outlying ALT values, and no inference can be made. No other correlations were evident. ALP, alkaline phosphatase; ALT, alanine transaminase; HPF, high power field.

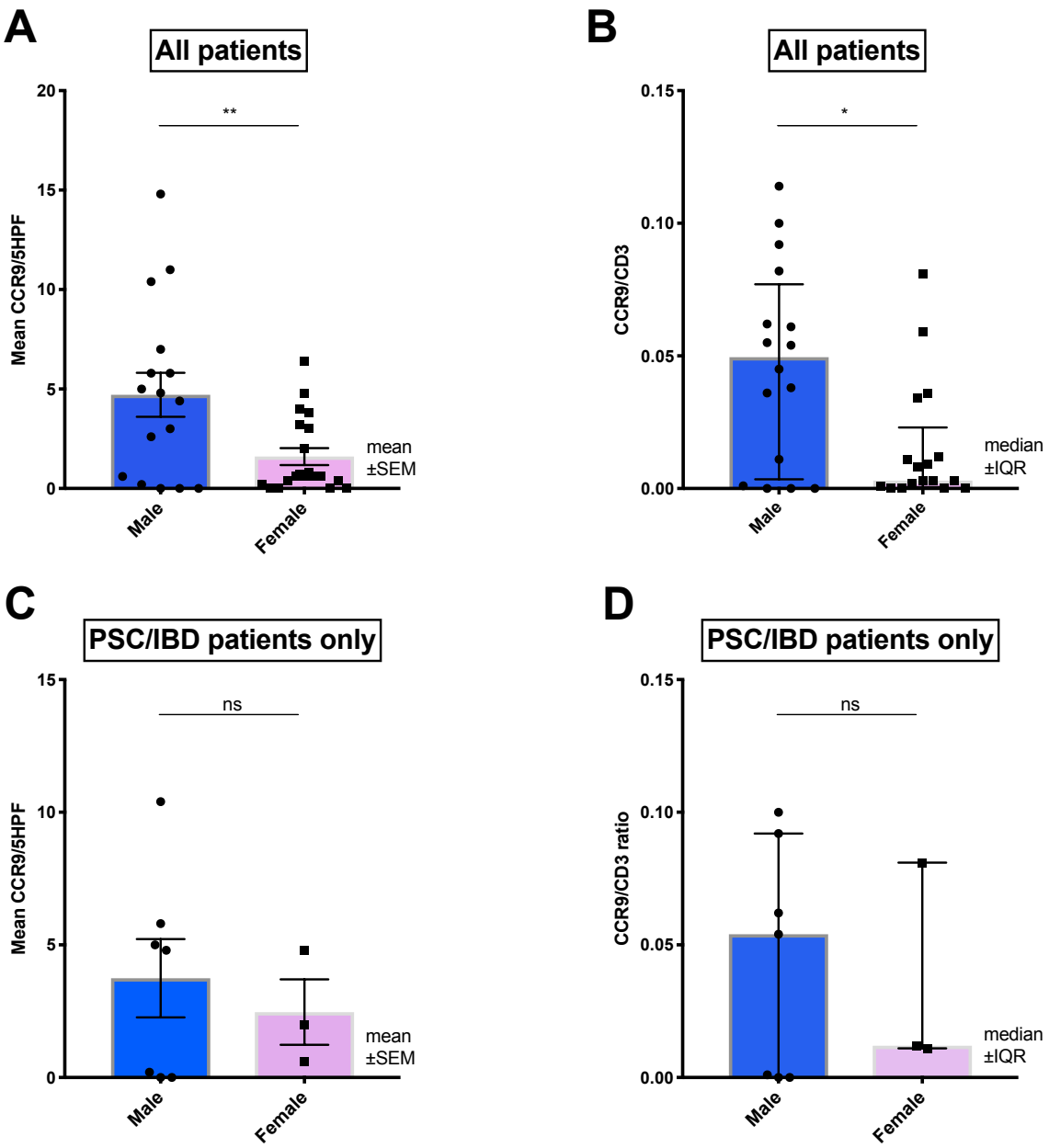


Figure 4-27 -The hepatic CCR9 counts and ratios according to gender

Males had a higher mean CCR9/5HPF (A) and median CCR9/CD3 ratio (B) on liver IHC compared with females when analysing the total cohort of patients with PSC/IBD, PSC/no IBD and LC (A and B). This was also true numerically when comparing only patients with PSC/IBD (C and D respectively). Unpaired t-tests or Mann Whitney tests were applied according to normal or non-normal distribution respectively. HPF, high power field; IBD, inflammatory bowel disease; LC, liver controls; PSC, primary sclerosing cholangitis. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

Because of this difference seen between males and females on IHC, I went back to the FNA data to compare CCR9 proportions between males and females.

In contrast to the IHC findings, females had a higher median proportion of CD4+ memory T-cells which were CCR9+ (5.3% [IQR 4.4-5.9]) compared to males (2.5% [IQR 2.2-3.8], p=0.014, see *Figure 4-2*). This was also true for the proportion of CD8+ memory T-cells which were CCR9+ (4.0% [IQR 1.8-6.0] vs. 1.5% [IQR 0.6-2.6], p=0.015, respectively). There was a slightly higher proportion of males within the PSC group (66.7%) compared with the LC group (52.9%), so the comparisons between males and females were also made within each disease cohort separately. These changes were numerically the same within each cohort, though did not quite reach statistical significance.

The two modalities of IHC on FFPE liver biopsies and flow cytometry on liver FNAs are not directly comparable for several reasons. Firstly, IHC only examined tissue-resident T-cells, whereas FNA sampled LILs which may be tissue resident and intrasinusoidal. Secondly, IHC examined mean total count of CCR9+/5HPF and proportion of CD3+ T-cells whilst analysis of FNA samples was on the proportion of CD4+ and CD8+ memory T-cells which were CCR9+. Thirdly, a different CCR9 monoclonal antibody was used between the two experiments which examine different epitopes, though the manufacturers declare good specificity for the CCR9 molecule. Lastly, the baseline demographics between the two experiments differed slightly (slightly older group and more NAFLD in the FNA group, younger and more autoimmune LCs in the IHC group).

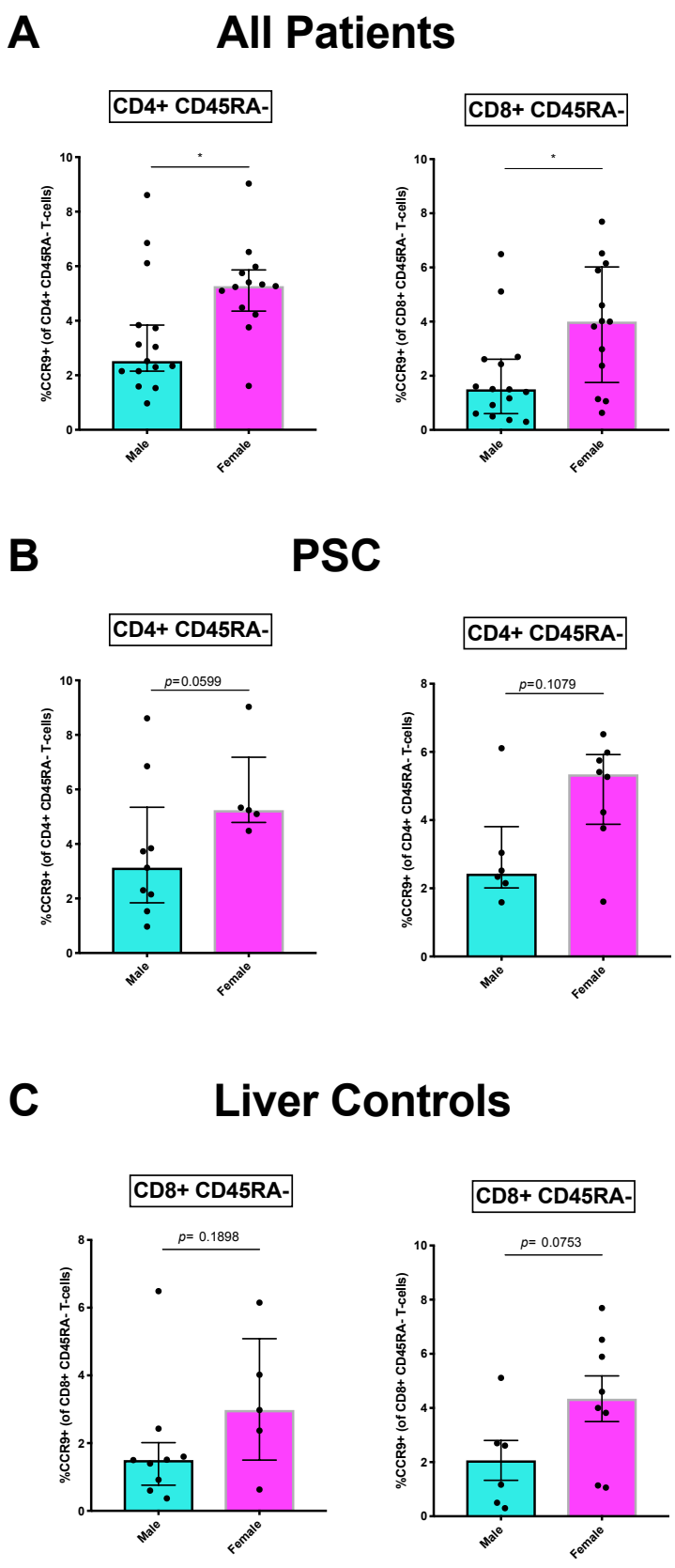


Figure 4-28 -Males versus females: flow cytometric analysis of CCR9 expression on CD4+ and CD8+ memory LILs derived from FNAs

Liver FNA was performed on patients with PSC and LC and LILs underwent cell surface antibody staining and flow cytometry analysis. The proportion of CD4+ and CD8+ memory T-cells which were CCR9+ were compared between males and females. A, The median proportion of CD4+ CD45RA- T-cells (left) and CD8+ CD45RA- T-cells (right) which were CCR9+ was higher in females compared with males. This was also true when analysing the disease cohorts separately: B, PSC; C, LC; though not to statistical significance. Graphs depict median values and IQR. For normally distributed values, unpaired t-test was applied, and for non-normally distributed values, Mann Whitney test was applied. p-values are indicated on graphs, or *=p<0.05. CCR9, CC-chemokine receptor 9; FNA, fine needle aspiration; LC, liver controls; LIL, liver-infiltrating lymphocytes; PSC, primary sclerosing cholangitis.

Perhaps there is a true intrahepatic difference where males have a higher proportion of CCR9+ liver-resident T-cells as demonstrated by IHC, potentially from increased sinusoidal endothelial expression of chemokines (CCL25) and adhesion molecules (VAP-1, MAdCAM-1) in males leading to increased recruitment. And this is demonstrated by a lower intrasinusoidal concentration of CCR9+ T-cells in males which may be reflected more by the FNA flow cytometry analysis. It would be interesting to evaluate the differences in the expression of these endothelial molecules between males and females within the liver sinusoidal compartment to test this hypothesis.

4.5 Discussion and summary of findings

In this chapter I have demonstrated a safe and effective technique (liver FNA) to sample LILs for immunophenotyping. Liver FNA has been demonstrated as an effective technique before in small subsets of patients, and these data presented here support its use in PSC which has not been done before. Furthermore, there is minimal data documenting the safety and tolerability of liver FNA,[190] and these results show that FNA is well tolerated, with minimal to no pain post-procedure, no severe adverse events if performed alone (i.e. no concomitant liver biopsy) and is supported by patients as demonstrated by the willingness of patients to undergo a second FNA.

Furthermore, the yield of lymphocytes and CD3+ T-cells from FNA is sufficient to derive meaningful results from flow cytometric and transcriptional analysis. However, it must be noted that on occasion, yields of FNA are too low, or the phenotype is too similar to PBMC, to allow adequate analysis. This was also found to be the case in the study by Gill *et al*.[196] They reported that any aspirate with frank blood staining was discarded but do

not report how often this occurred. In my experience, a reasonable proportion of FNAs (around a quarter) had a small amount of frank blood staining (e.g. 100-200μL) which may have affected the aspirate phenotype slightly more towards that of PBMC, but these samples were not discarded. Furthermore, Gill *et al*. also had a wide variation in the proportion of certain subtypes of cells (such as CD4+, CD8+, NK cells) and developed a tool to determine how "liver-like" an FNA was. They looked at the proportion of NK cells which expressed chemokine receptor, CXCR6, and the proportion of CD8+ T-cells which co-expressed CD69 and CD103 (i.e. T_{RM}). Whilst I did not look specifically at these parameters within this study, the fact that they developed such a tool acknowledges that there is some variation in the liver-specific nature of the aspirate as compared with PBMC.

I demonstrated that it is possible to sort CD45+ lymphoid/myeloid cells from liver FNAs and perform single cell RNAseq and identify a liver-specific cluster of cells. This cluster showed certain identifiers of NK cells, but a deeper analysis is required, and this will be done after interrogation at a higher resolution in an effort to discover further clusters.

I was not able to demonstrate that there is a higher proportion of gut-homing T-cells within the PSC liver as compared with LC, however I made some interesting observations. Firstly, I demonstrated that there are a subset of patients with underlying chronic liver disease who have proportionately high amounts of CD4+ memory T-cells which are CCR9+ (e.g. >5%, by flow cytometry on FNAs) and high amounts of CD3+ T-cells which are CCR9+ which reside around the portal tracts (e.g. >5CCR9+/5HPF, by IHC). There appeared in both experimental techniques to be two subsets of patients – those with low hepatic

CCR9+ T-cells and those with raised hepatic CCR9+ T-cells. The LC patients examined had a mix of diseases including NAFLD/NASH, PBC, AIH, and ALD. Previously, LILs have only been examined for CCR9 expression in PBC and PSC.[137, 138] Patients with the highest proportions of CCR9+ cells among CD4+ memory T-cells anecdotally had markers demonstrating high levels of fibrosis, though I could only show a trend for correlation of CCR9 expression with LSM. Nevertheless, it may be that rather than being a specific feature of PSC, the presence of CCR9+ T-cells within the liver may be a reflection of chronic inflammation and fibrosis. Indeed ongoing liver injury and inflammation causes an upregulation of chemokines and adhesion molecules which may promote recruitment of CCR9+ T-cells.

There are only two other studies which have examined for the presence of CCR9+ T-cells within the liver of patients with PSC.[137, 138] Both came from Birmingham, UK, were ten years apart, both derived LILs from liver explants, and controls in both cases were with PBC (and in one setting, also normal donor liver). In the earlier study by Eksteen *et al*. they reported that a mean 17% of PSC CD3+ LILs were CCR9+ compared with <2% of LILs from patients with PBC and healthy controls (via flow cytometry with a differing monoclonal CCR9 antibody from R&D Systems).[137] Furthermore, they reported that the majority of these CCR9+ T-cells were CD8+, though the data was not shown (which is not in keeping with my data). They included 8 patients with PSC, 8 with PBC and 8 healthy controls.

Meanwhile, Liaskou *et al*. included 5 patients with PSC and 2 patients with PBC.[138] They found that roughly 25% of CD4+ LILs in PSC were CCR9+ compared with roughly 10% in

PBC. The statistical significance was not reported, though the numbers were very small. They found lower expression of CCR9 on CD8+ LILs, similar to my findings, and no difference between PSC and PBC. The clone of CCR9 monoclonal antibody was not reported.

The proportions of T-cells which were CCR9+ are much higher than those found in my study. This is likely to be partly due to the nature of obtaining the LILs – they were dissociated mechanically from liver explants (similar to my resections technique) as compared with my sampling via FNA. Therefore, they were likely to have a higher proportion of liver-resident T-cells compared with my FNA technique. It is possible that the FNA technique is not liver-specific enough to pick up differences in populations which are rare, such as CCR9+ T-cells. It is also possible that in fact there is no difference in the proportion of LILs expressing CCR9 between PSC and LC as demonstrated by my data – I had almost double the number of patients with 14 PSC and 14 LC patients included in the FNA analysis.

The transcriptional analysis may reveal further differences within the liver between PSC and LC, particularly among the CD4+ T-cell cluster, as shown by the t-SNE plots. This can be explored further with interrogation at a higher resolution; however it is unlikely this will shed any more light on CCR9 differences between PSC and LC given the low numbers of CCR9 transcripts identified. Meanwhile, there was a trend towards a higher CCR9 count and ratio via IHC in PSC/IBD as compared with PSC/no IBD, and to a lesser extent as compared with LC.

Finally, I did demonstrate a trend towards a higher proportion of MAIT cells among CD8+ memory T-cells within the PSC liver compared with LC, which would be in keeping with the lower proportion seen in the peripheral blood in PSC compared with HC and UC. Whether MAIT cells play a pathogenic or protective role within the liver in PSC, however, is not clear. MAIT cells often can have a pro-inflammatory phenotype (T_H1/T_H17).[204] Furthermore, I found a higher proportion of activated CD8+ memory cells within the PSC liver compared with LC as demonstrated by higher proportions of CD69+ cells.

Taken together, it is difficult to draw a firm conclusion from these data and comparisons with the existing literature regarding to what extent gut-homing lymphocytes accumulate in the PSC liver but not in control livers. Therefore, the null hypothesis cannot be rejected and further analyses are needed - however the power of the FNA scRNAseq approach means these studies can now be applied to much larger patient (and relevant control) populations with a diverse range of disease stages.

5 The Clinical Effects of Vedolizumab in PSC/IBD

5.1 Introduction

Vedolizumab has been shown to be effective in IBD with regards to clinical response and clinical remission as seen in the GEMINI studies as well as real world data.[76, 205] Patients with PSC were not specifically mentioned in these registration studies, and there is little existing data on the effect of VDZ on patients with PSC/IBD. Furthermore, there is some evidence, both clinically and immunologically, that PSC/IBD has its own phenotype as distinct from UC or CD alone.[3, 83, 84] Indeed, data I presented in Chapter 3 found a difference in the proportion of CCR9+ T-cells in the ileum of patients with PSC/IBD compared with UC alone or HC. It is not clear how the IBD in PSC/IBD responds clinically to VDZ, and whether it has similar efficacy to IBD alone.

Secondly, despite the lack of a clear difference in the proportion of gut-homing lymphocytes in the liver in PSC versus controls in my own findings in Chapter 4, there is a suggestion that there is a subpopulation of patients with PSC who have high proportions of CCR9+ cells in the liver, as demonstrated by flow cytometry and IHC. Whether VDZ plays any role in interrupting this pathway is unclear. The reports of VDZ in patients with PSC is limited to only 5 publications and 2 abstracts with limited numbers of patients included.[41, 150-155] The data presented from these reports do not give a clear indication whether VDZ is effective upon the PSC/IBD, nor whether VDZ impacts on liver outcomes, as evidenced by changes in liver biochemistry and other more concrete liver outcomes such as liver transplantation.

5.1.1 Aim

Given these small amount of data and the clinical uncertainty as to the outcomes of VDZ in PSC, I sought to evaluate the effect of vedolizumab on patients with PSC/IBD with regards to changes in biochemistry and IBD response in our single centre patient cohort at Oxford.

5.2 Methods for Oxford vedolizumab study

5.2.1 Patient cohort

Patients with PSC/IBD who were prescribed VDZ for treatment of their IBD were prospectively identified at John Radcliffe Hospital, Oxford, UK. To be included, patients must:

- Have a diagnosis of PSC or small duct-PSC according to internationally recognised guidelines.[206, 207]
- Have an established diagnosis of IBD, including UC, CD or IBD-unspecified (IBD-u), consistent with recognised European guidelines.[208]
- Not have previously received a liver transplant.

VDZ was administered via the licenced induction dosing of 300mg intravenously at day 0, week 2 and week 6, and subsequent maintenance infusions of 300mg IV 8-weekly. VDZ was continued or discontinued depending on clinical outcome and/or adverse events, as determined by the patient's treating physician.

5.2.2 Data collected

General demographics were recorded including age and gender, type of IBD and type of PSC, concomitant medications at the start of VDZ, including immunomodulators, steroids and UDCA, and prior use of biologic agents. The patient's most recent liver elastography reading was also recorded.

Biochemical data collected included liver biochemistry comprising ALP, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin, as well as baseline serum albumin, prothrombin time (PT), platelets and creatinine. Liver biochemistry was performed at baseline as well as immediately prior to each infusion (day 0, day 14, day 42, day 96 etc) as well as at various time points in between for ad hoc clinical purposes.

Due to the poor correlation between symptoms and active inflammation in IBD, hard endpoints were chosen to assess the status of IBD at baseline and at follow-up on VDZ. These comprised an endoscopic assessment - UCEIS (range 0-8, with 8/8 most severe), and a histological assessment - NI (range 0-4, with 4/4 the most severe). These were determined from endoscopies (either flexible sigmoidoscopy or colonoscopy) performed at baseline (prior to VDZ induction) and at some time point whilst on VDZ, usually at about 4-6 months.

Liver-related outcomes collected included new onset ascites, cholangitis, variceal bleeding, cholangiocarcinoma, listing for liver transplantation (LT), and LT.

5.2.3 Outcomes

The effect of VDZ on progression of PSC was evaluated by analysing the overall change in ALP from baseline to various time points when on VDZ - day 42 and last follow-up whilst on VDZ. The time point of day 42 was used as an early time-point to assess change in liver biochemistry as this corresponds with the last induction dose of VDZ. In clinical practice this is often the time when initial clinical response of IBD to VDZ treatment is typically assessed.[145] Liver biochemistry results were included up until 56 days after the final VDZ infusion, as 56 days is the interval between VDZ maintenance doses. To allow for variation in clinical practice, laboratory test results from days 43-46 could be assigned as the day 42 value.

With regards to IBD outcome, I evaluated overall change in UCEIS and NI from baseline to follow-up as well as whether at follow-up endoscopy patients had achieved a clinical response or remission. A clinical response was defined endoscopically as UCEIS reduction from baseline of $\geq$2 (with baseline UCEIS $\geq$3), and histologically as NI reduction by $\geq$1 (with baseline NI $\geq$2). Endoscopic remission was defined as UCEIS $\leq$1 (with baseline $\geq$3) and histological remission was classified as Nancy $\leq$1 (with baseline NI $\geq$2).

The percentage change in ALP from baseline to last follow-up was compared between IBD responders and non-responders as well as between IBD remitters and non-remitters, both from an endoscopic and histologic viewpoint.

Finally, I collected liver related outcomes, which included any of the following: listing for LT, undergoing LT, ascending cholangitis, new onset ascites, variceal bleed, hepatic

encephalopathy, cholangiocarcinoma, and death. Data were collected until the last VDZ dose, whether that be at study close or at last dose before drug cessation, whichever came first.

5.2.4 Statistical analysis

In comparing continuous variables from baseline to a later time point, paired t-tests or Wilcoxon matched-pairs signed rank tests were used according to the whether the data was distributed parametrically or non-parametrically respectively. Contingency tables with Fisher's exact test were used to compare proportions. The statistical software used was GraphPad Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com).

5.3 Results for Oxford vedolizumab study

5.3.1 Baseline demographics

The baseline demographics are shown in *Table 5.1*. Eleven patients with PSC/IBD were eligible for the study and were followed on VDZ for a mean 288 days (SD 108, range 154-508, see

Table 5.1). All patients had UC, the median age was 25 years (IQR 21-29, range 19-48) and 10/11 were male. At the time of censorship, 7/11 patients were still on VDZ (mean 317 days, range 95-509). 4/11 patients had ceased VDZ after a mean 237 days (range 154-438), mostly due to an inadequate response (3/4) and one patient due to ongoing extraintestinal manifestation (arthropathy) despite a clinical response for the colitis. All of these 4 patients went on to alternative biological therapy.

Table 5.1 - Baseline demographics of Oxford PSC-IBD patients on VDZ (n=11)

Age, median (IQR)	25y (21-29)
Male, n (%)	10 (90.9%)
Type of IBD UC CD IBD-u	 11 (100%) 0 (0%) 0 (0%)
Baseline medications: UDCA, n (%) Corticosteroids, n (%) 5-ASA, n (%) Immunomodulator, n (%)	 7 (63.6%) 2 (18.1%) 7 (63.6%) 3 (27.3%)
Previous anti-TNF, n(%)	3 (27.3%)
Baseline ALP, median (IQR)	143 IU/L (100-233)
Baseline UCEIS, mean (±SEM, range)	4.4 (±0.5, 1-6)
Pancolitis, n (%)	11 (100%)

ALP, alkaline phosphatase; 5-ASA, 5-aminosalicylic acid; CD, Crohn's disease; IBD, inflammatory bowel disease; n, number; TNF, tumour necrosis factor; UC, ulcerative colitis; UCEIS, ulcerative colitis endoscopic index of severity; UDCA, ursodeoxycholic acid.

5.3.2 Changes in liver biochemistry

The baseline median ALP was 143 IU/L (IQR100-233) which rose to 196 IU/L (IQR85-305) at day 42 and fell to 119 IU/L (IQR66-274) at last follow-up (see *Figure 5-1*). The difference between the baseline ALP and both of these time points was not statistically significant. However, what was clinically apparent, was that there appeared to be a dichotomous ALP response. For patients whose ALP was normal at commencement of VDZ, their ALP followed a relatively stable course (and the last follow-up ALP remained below the upper limit of normal); whereas for those whose ALP at Day 0 was >ULN, most (5/6, 83%) had an initial ALP rise, and thereafter it followed a fairly erratic course (see Figure 5-2).

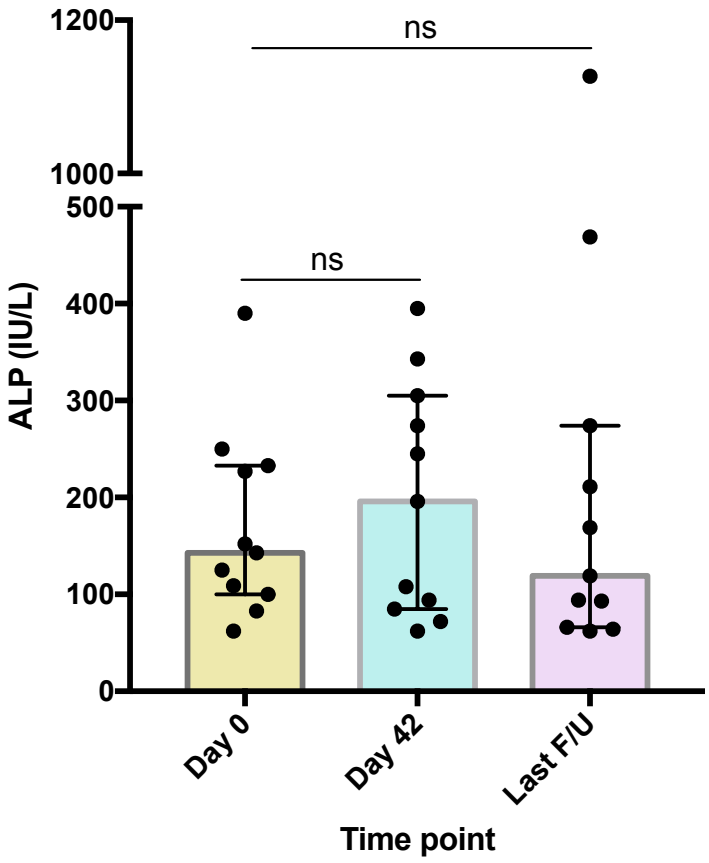


Figure 5-1 - ALP at baseline and at various timepoints post vedolizumab

Serum ALP was measured at baseline, day 42 and at last follow-up and the median value (+/- IQR) is shown. Blood was drawn immediately prior to vedolizumab infusion. Wilcoxon matched-pairs signed rank test performed.

ALP, alkaline phosphatase; IU/l, international units per litre; F/U, follow-up; ns, not significant (i.e. p>0.05).

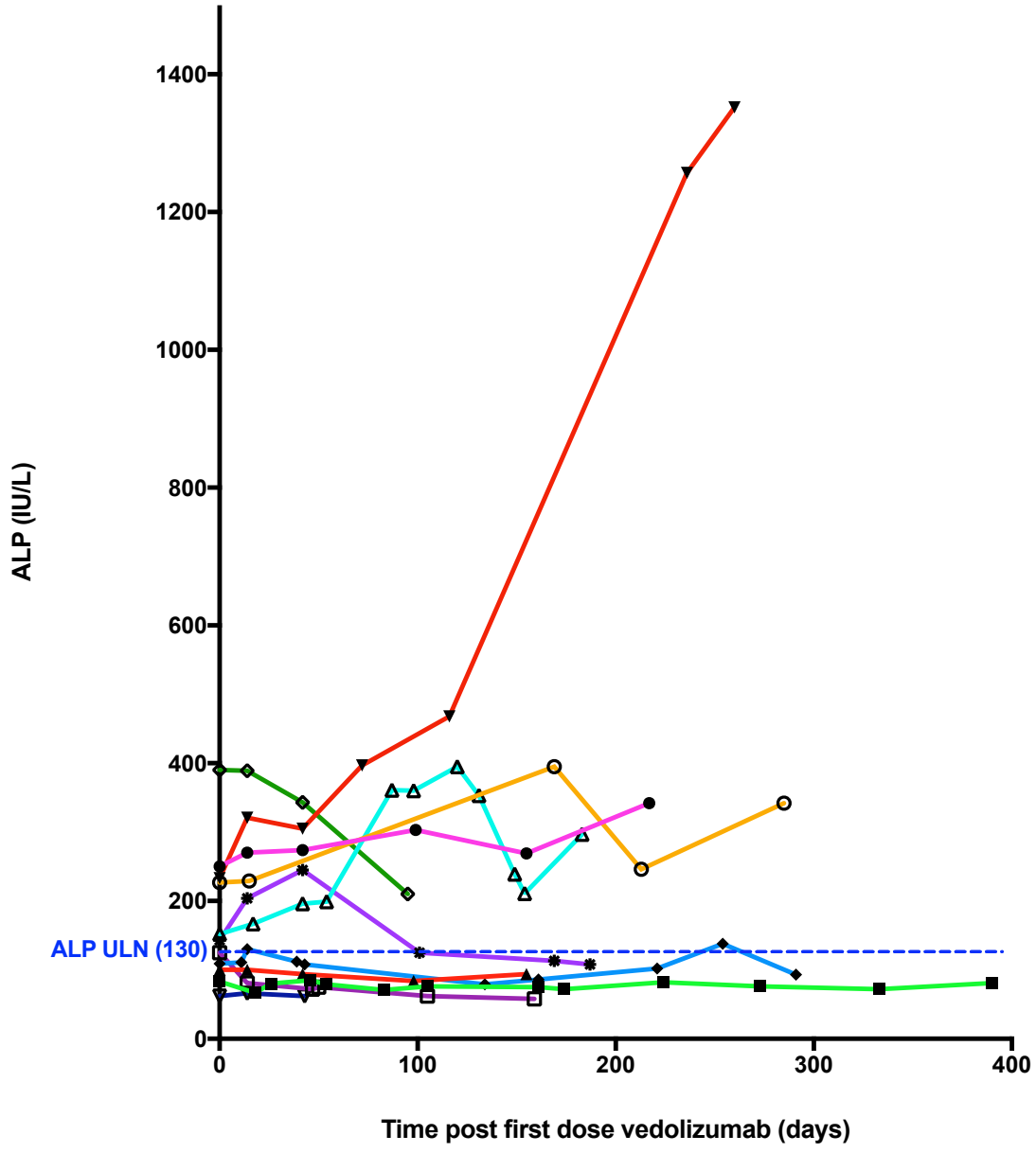


Figure 5-2 - ALP at various time intervals after starting vedolizumab

Each coloured line represents an individual patient (n=11) and shows the ALP measurement at various time-points while on vedolizumab. The dotted blue line represents the upper limit of normal for ALP. Patients whose baseline ALP was normal at baseline had a fairly stable course, compared with the fairly erratic course of ALP in those whose baseline ALP was >ULN.
ALP, alkaline phosphatase; IU/L, international units per litre; ULN, upper limit of normal.

5.3.3 IBD response

Endoscopic data was available for 10/11 patients. The index endoscopy was performed at a mean 36 days prior to VDZ initiation (range 3-99 days) and follow-up colonoscopy on VDZ was performed at a mean 201 days after the first dose of VDZ (range 86-473). UCEIS was calculated for all patients at both procedures.

There was a drop in mean UCEIS from baseline to follow-up colonoscopy: 4.6 (±SEM 0.5) vs. 2.4 (±SEM0.7, p=0.015, see Figure 5-3). Nine of ten patients had a UCEIS≥2 at baseline and therefore endoscopic response and remission could be assessed, and 7/10 patients had a NI≥1 at baseline, for assessment of histological response and remission. Five out of nine patients (55.6%) achieved an endoscopic response, with 4/9 (44.4%) achieving endoscopic remission. Similarly, 5/7 (71.4%) patients achieved a histological response, but only 2/7 (28.6%) achieved histological remission.

5.3.4 Association between liver biochemistry changes and IBD response

There was an association between endoscopic response and remission and proportionate ALP change from baseline to last follow-up, with a drop in ALP in responders/remitters, and a rise in non-responders/ non-remitters (see Figure 5-4). The median percentage ALP change in endoscopic remitters was -36.7% (IQR -55.1% to -16.8%) compared with +9.6% (IQR 0.25% to 211.3%, p=0.016), with a similar difference in endoscopic responders vs. non-responders.

Whilst there was a trend for a higher proportionate change in ALP in those who were histological remitters/responders, this did not reach statistical significance (See Figure 5-4).

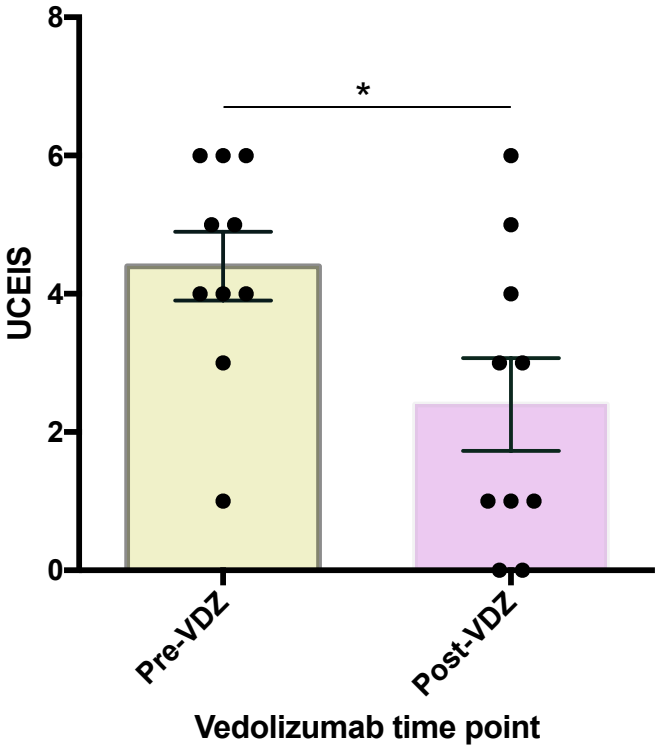


Figure 5-3 - UCEIS at baseline and at follow-up colonoscopy while on VDZ

The mean UCEIS dropped from baseline to follow-up colonoscopy after a mean 201 days post VDZ initiation.
UCEIS, ulcerative colitis endoscopic index of severity; VDZ, vedolizumab; * $p<0.05$.

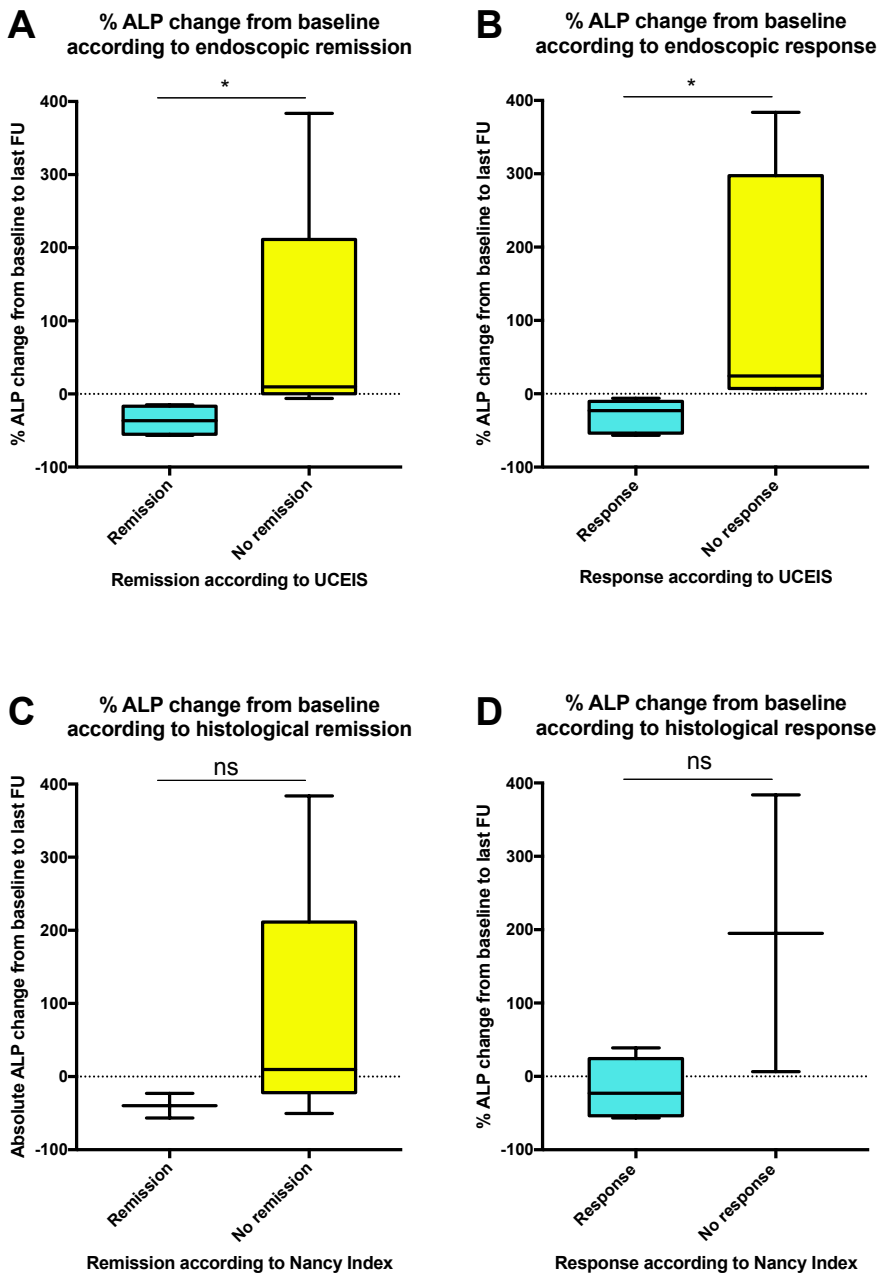


Figure 5-4 - Percentage change in ALP according to IBD response.

The ALP was assessed as a percentage change from baseline to last follow-up on VDZ, where a negative amount means a drop in ALP from baseline and a positive amount is a rise. Remitters/responders are shown in aqua and non-remitters/non-responders are shown in yellow. Median±IQR and range are shown. A&B: Endoscopic remission and response as classified by UCEIS (A: remission = UCEIS≤1; B: response = UCEIS drop ≥2); C&D: Histological remission as classified by NI (C: remission = NI≤1; D: response = NI drop≥1). Mann Whitney test performed.
ALP, alkaline phosphatase; NI, Nancy Index; UCEIS, ulcerative colitis index of severity; VDZ, vedolizumab. * $p<0.05$; ns= not significant.

5.3.5 Discontinuation from VDZ and Liver-related outcomes

Four patients (36.3%) discontinued VDZ at a mean 237 days (range 154-348). For 3/4, this was due to an inadequate response to VDZ, and for the other patient, it was due to ongoing active arthropathy, despite a successful IBD response. All four patients were switched to alternative biological therapy.

No patients suffered any liver-related clinical outcomes during the follow-up period. Four patients with liver enzyme abnormalities had a liver biopsy. None showed any sign of drug-induced liver injury, and none had their VDZ discontinued due to the liver enzyme elevation.

5.4 Interim discussion on Oxford cohort receiving VDZ

These preliminary data suggest that VDZ has no overall benefit in PSC/IBD with regards to ALP response but is effective in a substantial proportion of patients with respect to their IBD. The overall mean ALP did not change among 11 patients on VDZ at day 42 or at last follow-up, however it appeared that there was a dichotomous ALP deviation. Patients whose baseline ALP was normal (maybe due to the natural history of disease, or potentially due to the use of concomitant UDCA) had a stable ALP on VDZ without a rise to above the ULN at last follow-up. However, the erratic nature of the ALP in those whose baseline ALP was above the ULN may represent the natural history of the disease or may represent an effect of the VDZ, such as redirection of gut-homing lymphocytes from the bowel to the liver. This latter theory is not supported by the four liver biopsies performed in a subset of patients with liver enzyme rise.

Furthermore, patients who had an endoscopic response and/or remission had an overall ALP drop compared with non-responder/non-remitters, who had an overall ALP rise. This could in fact potentially be related to the intestinal isotype of ALP. It would make sense that patients with an IBD response might have a drop in intestinal ALP and those whose intestinal inflammation persisted had persistently raised intestinal ALP. However, the intestinal isotype makes up only a very small proportion of overall serum ALP, and isotype testing was not specifically performed in this study. There was an exception where one patient with an ALP elevation to the thousands had ALP isotypes evaluated, and in fact the ALP was predominantly hepatic.

If VDZ were blocking intestinal recruitment of gut homing lymphocytes and therefore redirecting them to the liver where tissue recruitment of lymphocytes is not as tightly regulated (and therefore may not be as susceptible to VDZ blockade), then the theory would follow that IBD responders were more likely to have an ALP rise than IBD non-responders. However, I have found the opposite is true.

One way to try and understand the effect of VDZ on the passage of $\alpha 4\beta 7$ T-cells through the body is to sample various tissue lymphocytes before and after VDZ. One could also compare whether this passage is related to IBD response or not. This will be explored further in the next chapter.

In any case, I felt that there was a heterogenous response to VDZ among these 11 patients with PSC/IBD, both with regards to ALP change and IBD response, but the sample size was too small to draw meaningful conclusions. I therefore sought to collaborate with

other centres across Europe and North America who are part of the International PSC Study Group (IPSCSG), to analyse a larger cohort.

5.5 Methods for international vedolizumab study

5.5.1 Patient cohort

A retrospective analysis was carried out on patients with PSC/IBD receiving VDZ as indicated for their IBD (UC, CD, or IBD-u). Investigators from 20 centres across Europe and North America who are active members of the International PSC Study Group (IPSCSG) contributed patient data (see *Table 5.2).* To be eligible for inclusion in the dataset, patients must have been diagnosed with PSC according to internationally accepted guidelines [207, 206]; have received a minimum of three doses of VDZ for their IBD; have baseline (pre-VDZ) and follow up blood tests including liver biochemistry; have commenced VDZ with their native liver still *in situ*; and received VDZ according to the usual dosing schedule as licensed.[209] A pro-forma detailing the inclusion and exclusion criteria, baseline demographics, and necessary data for collection was sent electronically to participating centres, and data was collated centrally (by myself). Efforts were made to collect missing data by electronic correspondence with participating centres. Patients for whom data were incomplete in respect of treatment or ALP at baseline and follow-up were excluded from the analysis. Endoscopic data were provided where available; however the lack of endoscopic scoring for IBD prior to the start of VDZ and at last follow up was not regarded as an exclusion criterion.

Table 5.2 - Participating Centres and the number of patients (n) from each centre included in the overall cohort (n=102).

Centre	Location	*n*
Oxford University Hospitals	Oxford, UK	15
Friedrich-Alexander-University	Erlangen, Germany	10
University of Alberta	Edmonton, Canada	10
Academic Medical Center	Amsterdam, Netherlands	7
University Hospitals Birmingham	Birmingham, UK	7
University Medical Center Hamburg-Eppendorf	Hamburg, UK	7
University of Miami	Miami, FL, USA	6
Massachusetts General Hospital	Boston, US	6
University of California Davis	Davis, CA, USA	6
Karolinska Instituet	Stockholm, Sweden	6
University of Washington Medical Center	Seattle, USA	4
Yale University School of Medicine	New Haven, CT, USA	4
Erasmus Medical Center	Rotterdam, Netherlands	4
University of Copenhagen	Copenhagen, Denmark	3
Medical University of Vienna	Vienna, Austria	2
California Pacific Medical Center	San Francisco, USA	1
University of Gothenburg	Gothenburg, Sweden	1
Ospedali Riuniti University Hospital	Ancona, Italy	1
University of Padova	Padova, Italy	1
Epatocentro Ticino	Lugano, Switzerland	1

5.5.2 Data collected

Baseline demographics collected included gender, age at diagnosis of PSC, type of PSC, presence of cirrhosis, type of IBD, race and ethnicity. The presence or not of cirrhosis was determined by the treating clinician according to clinical parameters, imaging and liver biopsy (where available). Whether VDZ had been discontinued was recorded, as was the reason for discontinuation (if known), the duration of VDZ treatment, baseline UDCA use and dose, and past use of anti-tumour necrosis factor (TNF) medication.

Clinical laboratory parameters collected at baseline and follow-up included serum ALP, ALT, AST, bilirubin and albumin, platelet count and international normalised ratio (INR). ALP was measured in international units per litre (IU/L) and calculated times the ULN. Follow-up laboratory tests were included up until LT, death, or 56 days following the final VDZ infusion (as this is the usual duration between maintenance infusions), whichever occurred first.

5.5.3 Outcomes

The effect of VDZ on progression of PSC was evaluated by analysing the change in liver biochemistry from baseline to various time points when on VDZ. This was done in two ways. Firstly, we analysed the overall change in ALP, ALT, AST and bilirubin levels at baseline, day 42, day 96 and last follow-up whilst on VDZ. The time points of day 42 and day 96 were used as early and medium time-points to assess change in liver biochemistry as these correspond with the last induction dose of VDZ and the first maintenance dose time-points. In clinical practice, the response of IBD to VDZ treatment is typically assessed after 6 to 10 weeks.[147, 145]. Liver biochemistry results were included up until 56 days

after the final VDZ infusion, as 56 days is the interval between VDZ maintenance doses. For these time-point analyses, only patient data that had paired baseline and the appropriate time-point data were used. To allow for variation in clinical practice, laboratory test results from days 30-59 could be assigned as the day 42 value, and from days 90-119 were used for the day 98 value.

Secondly, we deduced the proportion of patients whose ALP dropped by 20% or more from baseline to last follow up. This proportionate drop was chosen as it was judged to represent a drop of ALP of a larger magnitude than what would usually be considered to occur as part of the natural history of the disease and therefore considered clinically significant.[210] We also calculated the proportion of patients whose ALP rose by 20% from baseline to last follow up, and those whose ALP remained stable (i.e. +/-20%). Given the previous evidence for a worse prognosis in patients whose ALP is >1.5xULN,[159-161] we also evaluated within the subgroup of patients whose baseline ALP was >1.5xULN, the proportion whose ALP was <1.5xULN at last follow-up.

We sought to collect endoscopic IBD response, classified as improved, or unchanged/ worsened (as judged by the treating clinician), as well as endoscopic scores at baseline and on VDZ where available, namely MES,[51] the UCEIS,[52] and the Simple Endoscopic SES-CD.[53] We also looked at whether there was an association between endoscopic IBD response and change in ALP at last follow-up using the univariate analyses described below.

Finally, we collected liver related outcomes, which included any of the following: listing for LT, undergoing LT, ascending cholangitis, new onset ascites, variceal bleed, hepatic encephalopathy, cholangiocarcinoma, and death.

5.5.4 Statistical analyses

In comparing continuous variables from baseline to a later time point, paired t-tests or Wilcoxon matched-pairs signed rank tests were used according to the whether the data was distributed parametrically or non-parametrically respectively.

Univariate logistic regression was carried out for the outcomes of ALP reduction ≥20% from baseline to last follow-up, ALP rise ≥20% or more from baseline to last follow up, ALP<1.5xULN at last follow-up (within subgroup with baseline ALP>1.5xULN), the presence of liver-related outcomes, and improved endoscopic IBD response (versus worsened/ unchanged). Multivariate logistic regression was carried out to assess the impact of relevant variables on ALP drop or rise by 20% or more from baseline to last follow-up. Separate univariate and multivariate logistic regression was carried out on ALP drop by 20% or more for the cohort of patients who were not also on UDCA at VDZ initiation, as UDCA use can falsely lower ALP. Where there were missing covariates, a complete case analysis was carried out (see *Table 5.3*).

Table 5.3 - Number of variables missing within the complete dataset (n=102).

Variable	***n***
Presence of cirrhosis	1
Baseline ALP	0
Type of IBD	0
Gender	0
Age at diagnosis of PSC	0
UCDA use at baseline	0
Type of PSC	0
Duration vedolizumab	0
IBD response	28
Previous anti-TNF use	7
Paired baseline follow-up ALP	0
Paired baseline and follow-up ALT	1
Paired baseline and follow-up AST	34
Paired baseline and follow-up bilirubin	5
Baseline platelets	3
Baseline INR	39
Baseline albumin	9

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IBD, inflammatory bowel disease; INR, international normalised ratio; n, number; PSC, primary sclerosing cholangitis; TNF, tumour necrosis factor, UDCA, ursodeoxycholic acid.

The statistical software used was GraphPad Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com), and for the univariate and multivariate analyses, R 3.5.1 Core Team (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2018. URL http://www.R-project.org/.)

5.6 Results for international vedolizumab study

5.6.1 Baseline demographics

Of 133 patients whose data were contributed, 102 patients met inclusion criteria for the study. Reasons for exclusion were incomplete ALP data (n=15), first dose of VDZ received after liver transplantation (n=13) and less than three doses of VDZ administered (n=3). *Table 5.4* summarizes baseline demographics, clinical and laboratory information for the 102 study subjects: 64/102 (62.8%) were male, and the majority of patients had classical large-duct PSC (90.2%). One-fifth of patients had cirrhosis at baseline, and the majority of patients had associated UC (64.7%).

The median duration of VDZ treatment was 412 days (IQR 180-651, range 37-2609), and patients were followed up with regards to clinical endpoints for a median duration of 561 days (IQR 325-790, range 42-2614). All patients had received a minimum of three infusions at the usual dosing schedule, except for one patient whose third infusion was slightly earlier on day 37 rather than day 42. Forty-four patients (43.1%) had their VDZ treatment stopped during the study period. The majority of treatment cessation was due to lack of efficacy (32 patients, 72.3%), with six (13.6%) stopping due to an adverse reaction and another six stopping for unknown reasons.

Table 5.4 - Baseline Demographics, Clinical and Laboratory Data (n=102)

Male, n (%)	64 (62.8%)
Age at PSC diagnosis, mean (+/-SD, range)	31.4y (+/-14.2, 11-86)
Age at IBD diagnosis, mean (+/-SD, range)	26.0y (+/-12.3, 9-62)
Cirrhosis, n (%)	21 (20.6%)
Type of PSC, n (%)	
Large-duct PSC	92 (90.2%)
Small-duct PSC	8 (7.8%)
PSC/AIH overlap	2 (2.0%)
Type of IBD, n (%)	
Ulcerative colitis	66 (64.7%)
Crohn's disease	30 (29.4%)
IBD-unspecified	6 (5.9%)
UDCA use, n (%)	61 (59.8%)
Mean dose, mg/kg/day (+/-SD, range)	13.9 (+/-3.7, 5-20.4)
Previous anti-TNF use, n (%)	66 (64.7%)
Duration of vedolizumab, median (range)	412 days (37-2609)
ALP>ULN at baseline, n (%)	69 (67.7%)
Baseline labs, median (IQR)	
Alkaline phosphatase (ALP, IU/LxULN)	1.54 (0.86-2.67)
Alanine transaminase (ALT, IU/L)	38 (22-76)
Aspartate transaminase (AST, IU/L)	38 (23-69)
Bilirubin (umol/L)	10 (6.6-16.0)
Albumin (g/L)	37 (33-41)
Platelets ($x10^6$/L)	330 (213-401)
International Normalised Ratio (INR)	1 (0.97-1.12)
Creatinine (umol/L)	70 (63-80)
Sodium (umol/L)	139 (137-141)
AIH = autoimmune hepatitis; IBD = inflammatory bowel disease; PSC = primary sclerosing cholangitis; TNF = tumour necrosis alpha; UDCA = ursodeoxycholic acid.	

5.6.2 Changes in Liver Chemistry

In the total cohort, there was a small rise in liver enzymes over time (See *Figure 5-5*). The median ALP gradually rose from baseline (1.54xULN, IQR 0.86-2.67) to last follow up (1.64xULN, IQR 1.04-3.47, p=0.018), with similar to intermediate values at day 42 (1.55xULN, IQR 0.82-2.95, p=0.084) and day 98 (1.64 xULN, IQR 1.00-3.61, p=0.52). A comparable rise was seen for median ALT (baseline 38IU/L, IQR 22-76, vs. last follow up 53IU/L, IQR 29-100, p=0.002), median AST (baseline 38IU/L, IQR 23-69, vs. last follow up 49IU/L, IQR 27-93, p=0.0002) and median bilirubin (baseline 10umol/L, IQR 7-16, vs. last follow up 12umol/L, IQR 8-21, p=0.0002). The median duration post-VDZ of the last follow-up laboratory measurement varied depending on the biochemistry variable; ALP: 374 days (IQR 167-579); ALT 374 days (IQR166-613); AST: 438 days (IQR208-641); and bilirubin: 385 days (IQR 167-637).

Twenty-one (20.6%) patients had an ALP drop ≥20% from baseline to last follow-up. Thirty-nine patients (38.2%) had a stable ALP, whilst 42 patients (41.2%) had ALP rise by 20% or greater at last follow up *(*see *Figure 5-6).* The trajectories of the ALP from baseline over time are shown in *Figure 5-7.*

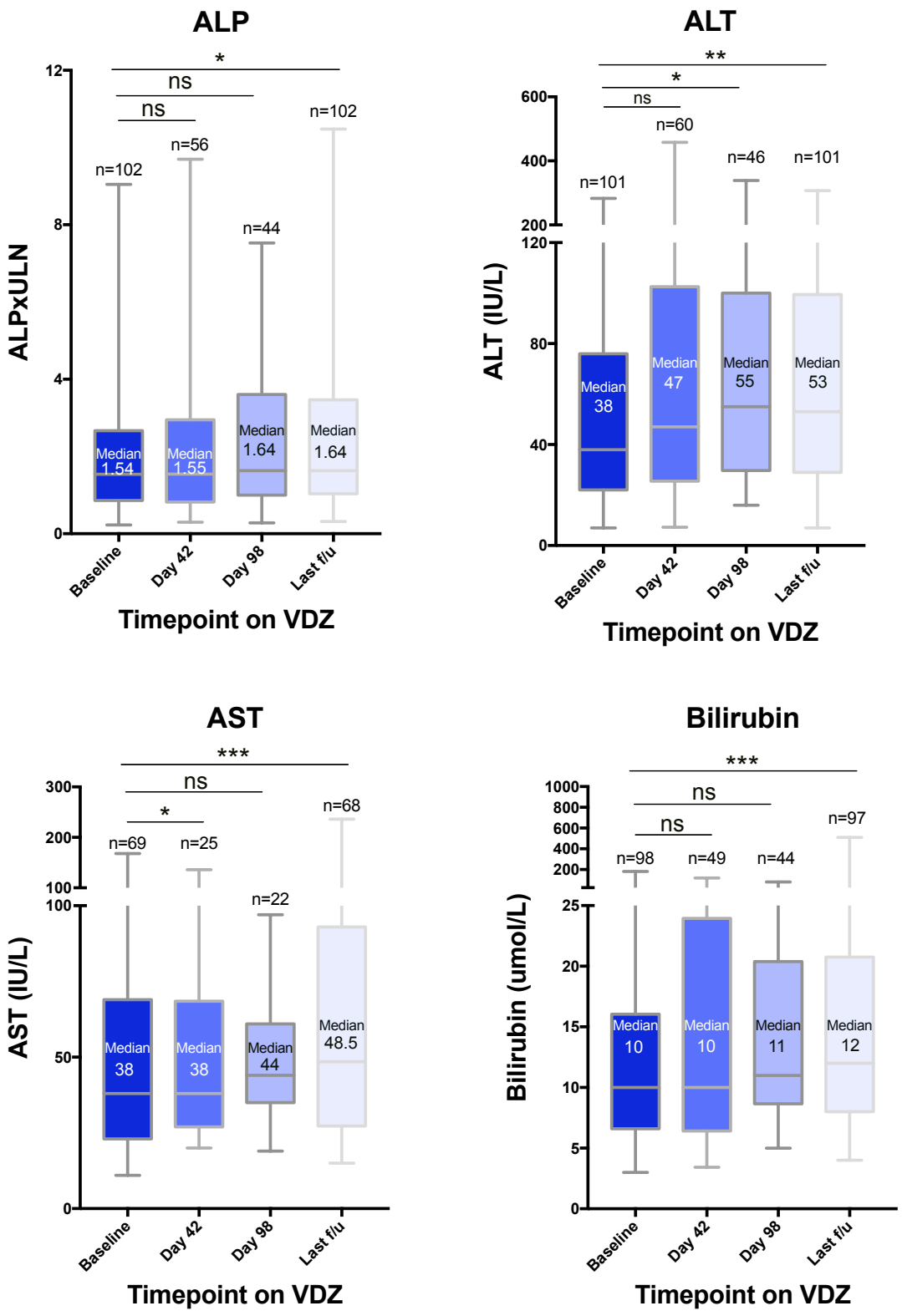


Figure 5-5 - Change in liver biochemistry over time on vedolizumab

The median, IQR and range are shown. The number of patients with liver biochemistry values and therefore included in the analyses are shown for each timepoint above the corresponding box and whiskers plot. The median duration post-VDZ of the last follow-up laboratory measurement varied depending on the biochemistry variable; ALP: 374 days (IQR 167-579); ALT 374 days (IQR166-613); AST: 438 days (IQR208-641); and bilirubin: 385 days (IQR 167-637). Wilcoxon matched-pairs signed rank test was performed. ALP=alkaline phosphatase; ALT=alanine aminotransferase; AST=aspartate aminotransferase; VDZ=vedolizumab; ns=p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001.

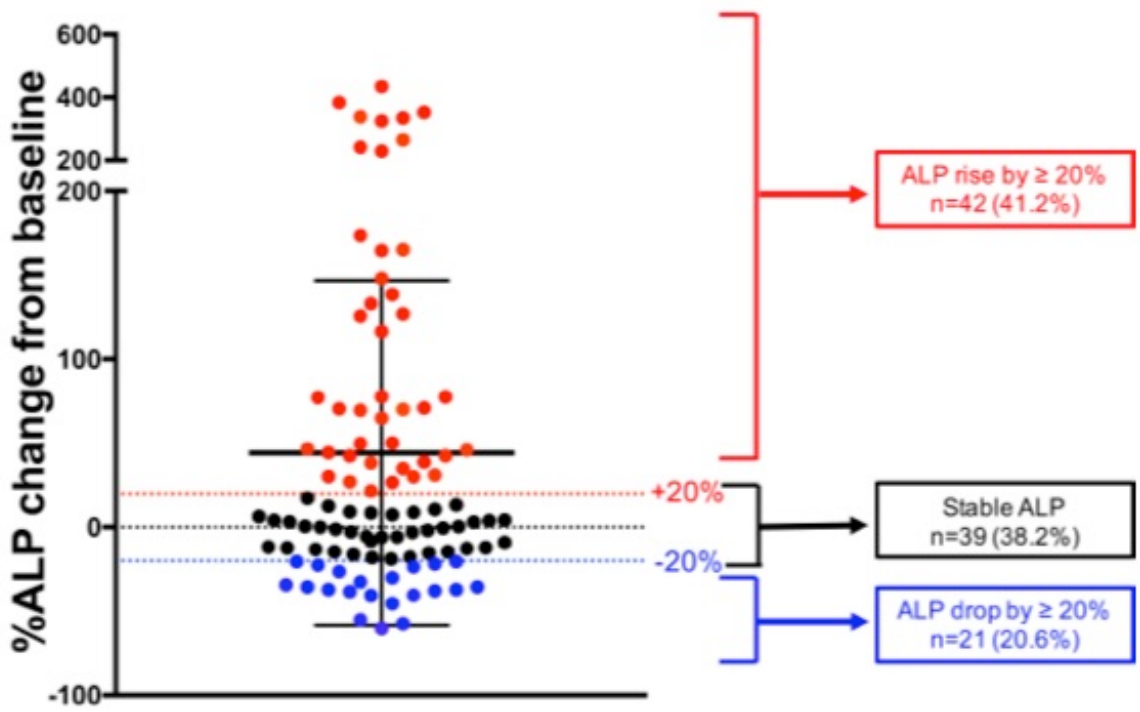


Figure 5-6 - The percentage change in ALP from baseline to last follow up.

Each dot represents an individual patient (n=102) and is colour coded to show three different groups. Red: ALP rise by ≥20%; Black: stable ALP (-20% to +20%); Blue: ALP drop by ≥20%. The black dotted line at 0 represents no change, with those below having a decrease in ALP at last follow-up and those above having an increase in ALP at last follow-up, as compared with baseline ALP prior to vedolizumab. ALP = alkaline phosphatase.

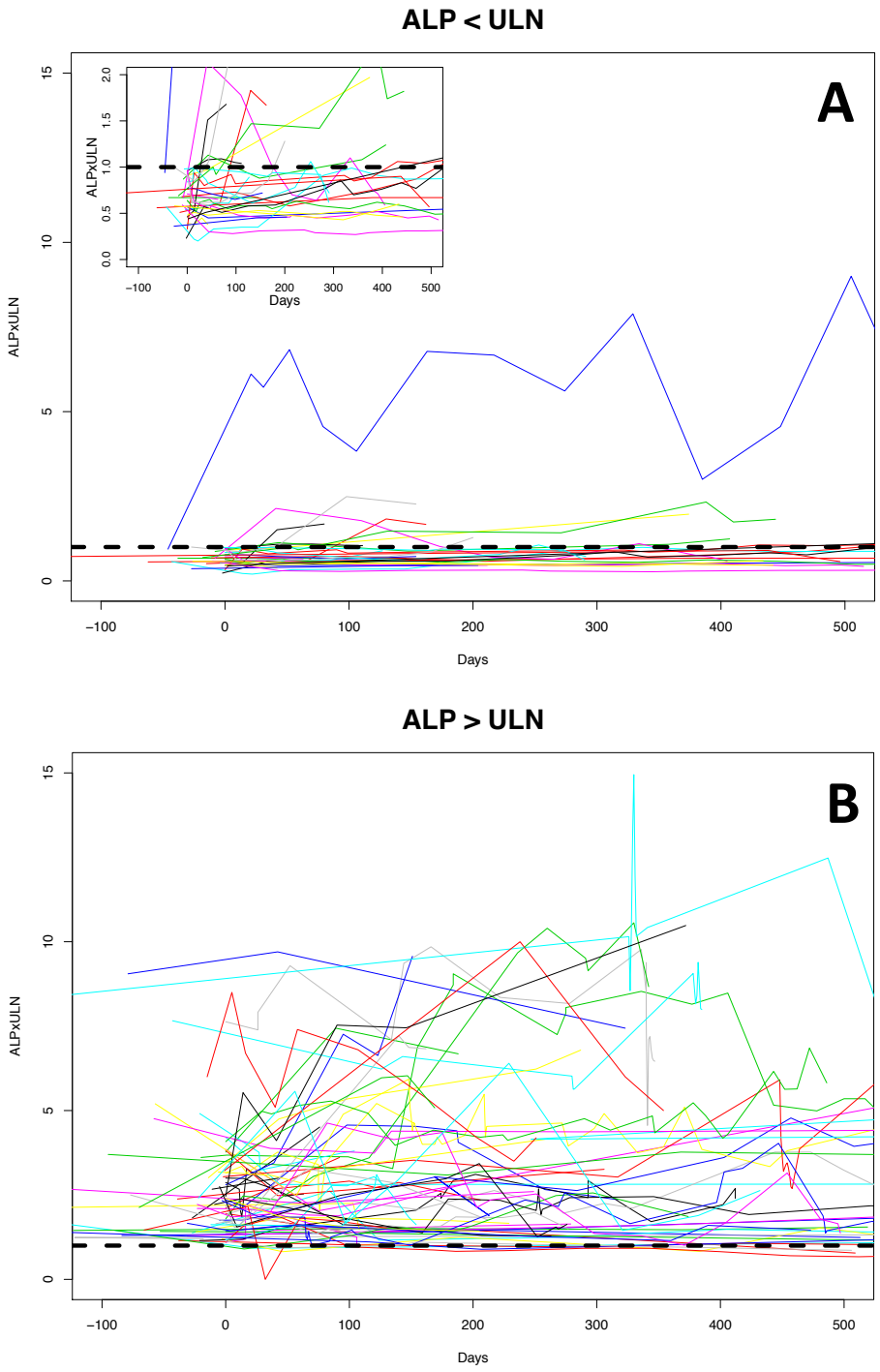


Figure 5-7 - The trajectory of ALP from baseline over time according to baseline ALP below and above ULN (n=102).

The trajectory of ALP from baseline over time according to baseline ALP below and above ULN (n=102). These spaghetti plots showing the ALP at baseline and different time-points on VDZ. Each line represents a unique patient. The dotted black line represents an ALP of 1xULN. (A) Patients whose baseline ALP was less than 1xULN; (B) Patients whose baseline ALP was greater than 1xULN. The inset in (A) is a magnified portion of the graph to more easily see the trajectory of those below the ULN. VDZ was started at day 0. Patients whose baseline ALP was <ULN tended to follow a fairly stable course, whereas those with baseline >ULN followed a fairly erratic course.
ALP, alkaline phosphatase; ULN, upper limit of normal; VDZ, vedolizumab.

On univariate analysis, the presence of cirrhosis was associated with an ALP drop of 20% or more from baseline to last follow up (OR 4.70, 95%CI 1.61-13.76, see *Table 5.5*). This finding was reproduced on multivariate analysis. No other variables were associated with ALP drop ≥20%, including UDCA use at baseline, although there was a trend towards an association a raised baseline ALP, and patients with Crohn's disease or IBD-u rather than UC . Twenty-nine percent of female patients and 42.9% of cirrhotics achieved such drop in ALP compared to 15.6% of males and 13.8% of non-cirrhotic patients. Of note, only 3 of the 21 patients with an ALP drop ≥20% had a normal ALP at baseline. No variables were associated with ALP rise ≥20% from baseline.

Separate univariate and multivariate analyses were carried out on the subgroup of patients who were not on UDCA at baseline (n=41, see *Table 5.6*). Ten patients (24.4%) had an ALP drop ≥20%; cirrhosis again was associated with this outcome in this cohort on univariate analysis, but not on multivariate analysis.

Table 5.5 - Univariate and Multivariate Analysis for ALP drop by 20% or more from baseline to last follow-up.

Variable	Univariate		Multivariate	
	Odds Ratio (95% CI)	**P-value**	**Odds Ratio (95% CI)**	**P-value**
Cirrhosis	4.70 (1.61-13.76)	0.005	4.48 (1.28-15.72)	0.019
Baseline ALP >ULN[a]	3.53 (0.96-12.98)	0.058	3.39 (0.76-15.25)	0.111
Ulcerative colitis[b]	0.37 (0.12-1.19)	0.096	0.35 (0.10-1.19)	0.092
Male gender	0.46 (0.17-1.20)	0.112	0.55 0.17-1.72)	0.301
Age at diagnosis of PSC[c]	1.03 (0.99-1.06)	0.111	1.01 (0.97-1.05)	0.723
UDCA use at baseline	0.68 (0.26-1.79)	0.438	0.55 (0.17-1.78)	0.318
Small duct-PSC[d]	0.55 (0.06-4.74)	0.585	-	
PSC-AIH overlap[d]	3.84 (0.23-64.29)	0.349	-	
Duration VDZ[e]	1.04 (0.98-1.09)	0.178	-	
IBD improvement on VDZ[f]	1.18 (0.37-3.75)	0.777	-	
Previous anti-TNF use	0.63 (0.22-1.83)	0.395	-	

[a]Baseline indicating last ALP taken before VDZ commenced; [b]versus IBD-unspecified or Crohn's disease; [c]per 1 year increase; [d]versus large duct-PSC; [e]per one month increase in vedolizumab duration; [f]endoscopic improvement versus unchanged/worsened.
ALP=alkaline phosphatase; AIH=autoimmune hepatitis; IBD=inflammatory bowel disease; PSC=primary sclerosing cholangitis; TNF=tumour necrosis factor; UDCA=ursodeoxycholic acid; ULN=upper limit of normal; VDZ=vedolizumab.

Table 5.6 - Univariate and multivariate analysis for ALP drop by 20% or more from baseline to last follow up among patients not on ursodeoxycholic acid (n=41).

Variable	Univariate		Multivariate	
	Odds Ratio (95% CI)	**P-value**	**Odds Ratio**	**P-value**
Cirrhosis	7.80 (1.60-38.11)	0.011	5.72 (0.68-47.79)	0.107
Baseline ALP >ULN[a]	2.83 (0.62-13.04)	0.181	2.12 (0.27-16.34)	0.472
Ulcerative Colitis[b]	0.24 (0.05-1.10)	0.067	0.41 (0.06-2.92)	0.370
Male gender	0.42 (0.10-1.81)	0.245	0.42 (0.06-3.03)	0.392
Age at diagnosis of PSC[c]	1.06 (1.00-1.12)	0.053	1.03 (0.95-1.11)	0.468
Small duct-PSC[d]	1.75 (0.14-21.88)	0.664	-	
PSC-AIH overlap[d]	3.50 (0.20-62.42)	0.394	-	
Duration vedolizumab[e]	1.06 (0.98-1.14)	0.157	-	
IBD improved[f]	0.75 (0.14-4.13)	0.741	-	
Previous anti-TNF use	0.83 (0.19-3.75)	0.812	-	

[a]Baseline indicating last ALP taken before VDZ commenced; [b]versus IBD-unspecified or Crohn's disease; [c]per 1 year increase; [d]versus large duct-PSC; [e]per one month increase in vedolizumab duration; [f]endoscopic improvement versus unchanged/worsened.
ALP=alkaline phosphatase; ULN=upper limit of normal; PSC=primary sclerosing cholangitis; AIH=autoimmune hepatitis; IBD=inflammatory bowel disease; TNF=tumour necrosis factor.

When analysing the subgroup of patients whose ALP was >1.5xULN at baseline, 10/51 (19.6%) had ALP<1.5xULN at last follow-up. Univariate analyses revealed that only type of IBD was associated with this outcome, with an OR of 0.07 (95% CI 0.013-0.39, p=0.002) for UC as compared with CD/IBD-u.

5.6.3 Endoscopic IBD response

Data on endoscopic response to VDZ at baseline and at follow up was available for 74 patients. The median duration from VDZ initiation to follow up endoscopy was 227 days (IQR 130-336, range 67-2540). Forty-two patients (56.8%) had an endoscopic IBD response, with the remainder worsened or unchanged. Normal ALP at baseline and longer VDZ duration were associated with an endoscopic IBD response, though type of IBD was not associated (see *Table 5.7*).

Endoscopic scores were available for a subset of patients (see *Figure 5-8*). Among patients with UC, the mean endoscopic scores dropped pre- versus post-VDZ for Mayo Endoscopic Subscore (2.2 [±SEM 0.8] to 1.4 [±0.9], p=0.0008) and UCEIS (4.2 [±1.5] to 2.5 [±2.0], p=0.028). Among patients with Crohn's disease, SES-CD pre- and post-VDZ was only available for five patients and there was a numerical improvement in mean SES-CD (17.0 [±9.1] to 8.8 [±11.0], p=0.215).

Table 5.7 - Univariate Analysis for Endoscopic IBD Response (n=74).

Variable	Univariate	
	Odds Ratio (95% CI)	**P-value**
Cirrhosis	0.79 (0.27-2.35)	0.677
Baseline ALP >ULN[a]	0.29 (0.10-0.85)	0.024
Ulcerative Colitis[b]	1.01 (0.39-2.61)	0.979
Male gender	1.12 (0.43-2.90)	0.815
Age at diagnosis of PSC[c]	1.01 (0.97-1.05)	0.635
UCDA use at baseline	2.57 (0.97-6.81)	0.058
Small duct-PSC[d]	1.15 (0.18-7.35)	0.880
PSC-AIH overlap[d]	n/a[e]	n/a
Duration vedolizumab[f]	1.08 (1.02-1.14)	0.013
Previous anti-TNF use	1.47 (0.53-4.08)	0.456

[a]Baseline indicating last ALP taken before VDZ commenced; [b]versus IBD-unspecified or Crohn's disease; [c]per 1 year increase; [d]versus large duct-PSC; [e]There were too few numbers of events to fit the binomial generalised linear model, and therefore an OR could not be calculated [f]per one month increase in vedolizumab duration.
ALP=alkaline phosphatase; ULN=upper limit of normal; PSC=primary sclerosing cholangitis; UDCA=ursodeoxycholic acid; AIH=autoimmune hepatitis; IBD=inflammatory bowel disease; TNF=tumour necrosis factor.

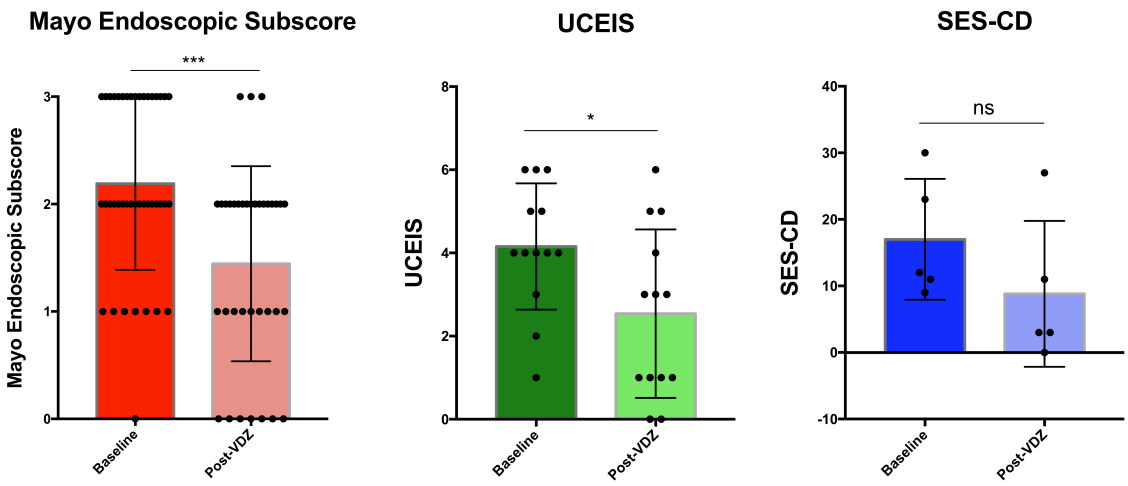


Figure 5-8 - Endoscopic IBD response at baseline endoscopy prior to VDZ and after treatment

Paired endoscopic scores were available for 36 patients for Mayo Endoscopic Subscore, 13 patients for UCEIS, and 5 patients for SES-CD. Individual scores for each patient are shown as black dots, with bars indicating the mean value and the standard deviation is shown. Paired T-test performed. UCEIS = Ulcerative Colitis Endoscopic Index of Severity; SES-CD = Simple Endoscopic Score for Crohn's Disease; VDZ = vedolizumab; ns=p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001.

5.6.4 Safety and liver-related outcomes

A 3-fold elevation in ALP, ALT and AST from baseline to last follow-up was observed in 6 (5.9%), 10 (9.9%) and 3 (4.4%) of patients; doubling of bilirubin was noted in 19 (19.6%). Twenty-one patients (20.6%) experienced a liver-related outcome over the median follow-up period of 561 days. Eleven patients (10.8%) were listed for LT, of whom 7 (6.9%) underwent LT. Nine patients (8.8%) experienced at least one episode of cholangitis and 6 patients (5.9%) had new-onset ascites. No patient experienced a variceal bleed, nor developed cholangiocarcinoma, and there were no deaths. On univariate analysis, cirrhosis, baseline ALP > ULN, and baseline albumin level were associated with the occurrence of a liver-related outcome (see *Table 5.8*).

*Table 5.8 - Univariate analysis for liver-related outcome (n=102)**

Variable	Odds Ratio	P-value
Cirrhosis	5.70 (1.96-16.57)	0.001
Baseline ALP >ULN[a]	1.67 (1.23-2.28)	0.001
Ulcerative Colitis[b]	1.12 (0.40-3.08)	0.833
Male gender	1.63 (0.57-4.65)	0.358
Age at diagnosis of PSC[c]	0.99 (0.96-1.03)	0.696
Baseline serum albumin[a,d]	0.90 (0.83-0.98)	0.024
Baseline Platelets[a,e]	0.96 (0.92-1.00)	0.051
Baseline INR[a,f]	1.28 (0.84-1.95)	0.251

*Liver-related outcome defined as any of the following: listing for LT, undergoing LT, ascending cholangitis, new onset ascites, variceal bleed, cholangiocarcinoma, and death. [a]Baseline indicating last lab value taken before VDZ commenced; [b]versus IBD-unspecified or Crohn's disease; [c]per 1 year increase; [d]per 1mg/L increase; [e]per 10 x 10^9/L increase; [f]per 0.1 units increase.
ALP=alkaline phosphatase; ULN=upper limit of normal; PSC=primary sclerosing cholangitis; IBD=inflammatory bowel disease; INR=international normalised ratio; LT=liver transplantation.

5.7 Discussion for international vedolizumab study

These data presented here, which represent an international, multicentre experience, adds substantially to the existing literature on the subject of patients with PSC exposed to VDZ. The demographics of the cohort closely resembled that reported in the literature, with the majority having large duct-PSC, two thirds having UC, and a third CD or IBD-u, a proportion having advanced liver disease, and two-thirds being on UDCA treatment.[211, 210] The median age at PSC diagnosis of 31.4 years is slightly lower than in the literature (40 years)[6] which may reflect the demographic who have more active IBD or are more likely to receive a biologic therapy. However, even though there are similarities between this cohort and that reported in the literature, one should keep in mind that this cohort has active IBD, which in itself is a minority of the PSC demographic, as most patients with PSC have fairly quiescent IBD, not requiring biologic therapy (or, 20-40% do not have IBD at all).[3] Therefore, the effect of VDZ in PSC with inactive IBD or no IBD has never been observed or evaluated, and warrants further investigation.

It is immediately apparent that there is heterogeneity with regard to changes in ALP, an important marker of cholestasis. The reduction in ALP of ≥20% from baseline in a subset of treated patients may indicate that there is a biological effect of VDZ in patients with PSC, although a more pronounced spontaneous fluctuation cannot be excluded.

ALP is thought to be a surrogate marker for clinical outcome in PSC, and has been proposed by a panel of experts as an important endpoint in clinical trials.[212] Although spontaneous normalization of ALP has been described in a variable proportion of patients with PSC irrespective of endoscopic intervention or use of UDCA,[213] the natural history

of PSC is for the ALP level to remain relatively stable or increase slightly over time. For instance, in a recent clinical trial investigating the role of nor-ursodeoxycholic acid on patients with PSC, the 40 patients on placebo had a mean relative change of 1.2% increase over the 12-week study duration.[210] Therefore, the observed reduction in ALP by 20% or more in a subset of patients on VDZ suggests a possible therapeutic signal worthy of additional investigation. However, a drop in ALP does not necessarily correlate with improved clinical outcome. As seen in the high dose UDCA trial for patients with PSC, those on UDCA experienced a significant drop in ALP as compared with placebo, but developed more liver-related clinical end-points.[32]

In the cohort overall, the median values for ALP, AST, ALT, and bilirubin all rose from baseline through to last follow-up on VDZ. This rise, though, was small, and may reflect changes commensurate with the natural history and progression of the disease. Median ALP rose from 1.54xULN to 1.64xULN, which is equivalent to 184 IU/L to 197 IU/L (if ULN = 120 IU/L). This is itself a clinically insignificant rise, and therefore, when viewed overall, the rise in ALP is unlikely to represent a significant safety signal.

The drop in ALP of ≥20% was associated with cirrhosis and there was a trend towards an association with a higher ALP at baseline. The presence of cirrhosis was determined by the treating clinician on clinical grounds, imaging and/or liver biopsy. Cirrhotic patients and those with a raised ALP are potentially more likely to have a more inflammatory and progressive form of PSC, and therefore may theoretically benefit more substantially from an anti-inflammatory effect of reduced migration of lymphocytes to the liver. Furthermore, VDZ is degraded to smaller peptides and amino acids in order to be

excreted by the kidneys, and this process is partially done by hepatic proteolytic degradation.[162] Cirrhosis may reduce/slow the breakdown of VDZ thereby leading to higher serum concentrations, possibly leading to greater clinical effect.

Christensen *et al.* also observed an association of an elevated ALP at baseline with subsequent reduction after treatment with VDZ.[153] In their study, when examining patients with raised ALP at baseline, 11/18 patients (69%) had an ALP drop, with median ALP going from 475 IU/L at baseline to 322.5 IU/L at week 14 and 283 IU/L at week 30. In patients with normal ALP at baseline, there was a trend for the ALP to rise slightly. Equally, these findings should be interpreted with caution, as statistical regression to the mean may account for the association with a higher ALP at baseline, and we were unable in this retrospective study to distinguish hepatic, intestinal, and other isoforms of ALP.

One fifth of our cohort (21/102) experienced a liver-related outcome. This may be slightly over-represented by the occurrence of cholangitis (9/102) which in itself, unless recurrent and refractory to oral antibiotics, it not necessarily an indication of advanced liver disease requiring liver transplantation. If we exclude those with cholangitis only (7/9), then 14/102 (13.7%) experienced a liver-related outcome. These findings are similar to the recently reported trial of anti-LOXL-2 antibody, simtuzumab, in PSC, where 47/234 patients (20.1%) experienced a PSC-related event, with an over-representation of cholangitis (n=31).[214] This was regardless of assignment to simtuzumab or placebo arms, and over a follow-up of 96 weeks (672 days), only slightly longer than our median follow-up duration of 561 days. This proportion of liver-related outcomes is consistent with the natural history of PSC and does not by itself indicate that VDZ treatment is

harmful in PSC. Furthermore, although a direct comparison is not possible, the rate of VDZ discontinuation in this cohort (43%) does not appear much different from that reported in real-world experience on IBD-only patients (35%).[205]

It is important to highlight the fact that a significant proportion of patients had an endoscopic IBD response, both as reported by the treating clinician (42/74 patients (56.8%)) as well as by the more robust measure of drop in individual endoscopic scores (particularly Mayo Endoscopic Subscore and UCEIS). Such a response rate is similar to that found in IBD alone.[215, 76] Given the possibility that PSC-IBD represents an immunologically and phenotypically distinct form of IBD from IBD alone,[3, 216, 217, 84] this finding is reassuring for clinicians considering VDZ to treat active IBD in patients PSC.

A major limitation of this study is its retrospective nature. The use of objective outcome measures such as liver biochemistries and endoscopic scores serve to minimise bias, however, adverse events, particular those not leading to hospitalization, death, or liver transplantation could potentially be under-reported. Additionally, liver biochemistry values were not available at all time points for all patients, and there was no pre-defined time point for the last follow-up, which introduces heterogeneity in the cohort. The absence of a comparator group such a placebo-treated, or matched control cohort is a weakness that disallows attribution of causality. Nonetheless, these data may provide an impetus to investigate the role of intestine-homing lymphocytes in PSC in more detail, potentially through a placebo-controlled trial with suitable collection of tissue and blood samples to explore the mechanism of action of VDZ in this otherwise neglected and serious illness. This is especially pertinent as a more versatile and attractive route of VDZ

administration, a subcutaneous formulation, is currently under evaluation (NCT02620046, NCT02611830, NCT02611817).

In summary, this large international experience of VDZ in patients with PSC/IBD shows that a clear-cut biochemical response to VDZ is not observed across the entire cohort. However, a subset of patients had a substantial drop in their ALP of 20% or more. Patients with more aggressive disease, such as the presence of cirrhosis and potentially those with a raised ALP at baseline were more likely to respond. In addition, the proportion of patients experiencing a liver-related outcome appears to be in keeping with the natural history of disease. Furthermore, more than half of PSC/IBD patients treated with VDZ had an endoscopic IBD response, similar to rates reported in IBD-only patients, and without an apparent increase in the discontinuation rate. Despite the disappointment with lack of a uniform response, further evaluation of VDZ as a beneficial treatment in PSC may be warranted in a subset of patients via a stratified randomised clinical trial.

5.8 Discussion and conclusions

Both studies – the smaller Oxford study of 11 patients and the international collaboration of 102 patients (which included the 11 initial Oxford patients) – showed a rise in overall ALP over time in patients with PSC/IBD given vedolizumab, the latter to statistical significance. Both studies, however, also showed a heterogenous response, where some patients had minimal change in ALP, some had an ALP rise, and some had a drop.
The smaller Oxford study indicated that an endoscopic IBD response and/or remission was associated with a drop in ALP, whereas the larger cohort found no association. The statistics used were slightly different, in that in the larger cohort, univariate and

multivariate logistic regressions were used (as opposed to Mann Whitney test in the smaller Oxford cohort). The more stringent measure is more likely the multivariate regression, and so it may be that there is no association between IBD outcome and ALP response at all. However, a more robust definition was used for the smaller Oxford study, with hard endpoints of remission according to endoscopic score. Therefore, the possibility remains that an IBD response or remission may be associated with an ALP drop in patients with PSC/IBD given VDZ, and these parameters should be analysed and investigated in any future prospective study of VDZ in PSC.

Cirrhosis was found to be associated with a higher odds of an ALP drop >20% and there was a trend towards an association of raised ALP at baseline. These were not analysed in the smaller Oxford cohort.

Overall, it appears that patients with PSC/IBD have a good response of their IBD to VDZ, and a proportion of patients have an ALP drop and a proportion have an ALP rise. Delineating the passage of gut-homing lymphocytes in PSC upon therapy with VDZ may be helpful in helping to delineate this heterogenous clinical response, and will be examined in the next chapter.

6 The Effect of Vedolizumab on Gut-homing T-cells

6.1 Introduction

There are limited data on the effect of VDZ on the distribution of gut-homing lymphocytes throughout the human body. The hypothesis is that VDZ binds to $\alpha4\beta7$ on gut-homing T-cells thereby preventing passage of these cells into the lamina propria and reducing intestinal inflammation. The data to support this mechanism of action, however, are lacking. There are a small number of studies which have evaluated the effect of VDZ upon the proportion of circulating $\alpha4\beta7$+ T-cells with conflicting results – some suggest a drop in these cells, whereas some report a rise, presumably due to their being prevented to leave the circulation and enter the gut.[164, 166, 165, 117, 167] Meanwhile, there have been attempts to evaluate biomarkers for clinical response to VDZ but with conflicting results.[166, 165, 117, 167]

There are no studies which have examined the effect of VDZ on specifically $\alpha4\beta7$+ T-cells in the human intestine, and there are only two studies which have evaluated the effect on human T-cells within the colon.[169, 171] One study suggested there was no reduction in T-cells within the colon after administration of VDZ. In contrast, a second study suggested a reduction in adoptively transferred human T-cells trafficking to the murine inflamed colon after VDZ.

There have been no studies which have evaluated the effect of VDZ on the trafficking of gut-homing T-cells to the liver. This is especially relevant for patients with PSC/IBD, where I have shown in Chapter 5 that there are varying changes in liver biochemistry with VDZ. Overall, there is a rise in ALP and ALT in patients with PSC/IBD receiving VDZ. However, a

small proportion of patients have a significant drop in their ALP, and a further proportion have an ALP rise, whilst the majority have negligible ALP changes.

6.2 Aims and hypotheses

I sought to identify the changes in $\alpha4\beta7$ expression among T-cell populations in peripheral blood, ileum and colon in patients with colonic IBD (predominantly UC), both with and without PSC, after administration of VDZ. I aimed to identify differences between PSC/IBD and IBD alone, as well as between baseline $\alpha4\beta7$ expression and $\alpha4\beta7$ changes in remitters versus non-remitters. Finally I aimed to evaluate for the presence of $\alpha4\beta7$+ cells in the livers of patients with PSC/UC after administration of VDZ.

My hypotheses were as follows:

- that total $\alpha4\beta7$ expression among CD4 and CD8 T-cell populations would decrease in the peripheral blood, due to internalisation of the receptor upon binding of VDZ, but that proportions of $\alpha4\beta7$+ CD4+ memory T-cells would rise within the peripheral blood due to being prevented from entering the gut.
- that $\alpha4\beta7$+ T-cells would reduce within the ileum and colon with VDZ therapy, and to a greater degree in remitters versus non-remitters.
- that remitters would have a higher expression of baseline $\alpha4\beta7$ in peripheral blood and tissue compared with non-remitters
- that proportionate rise with VDZ of $\alpha4\beta7$ expression on CD4+ memory T-cells in the peripheral blood would correlate with decreasing UCEIS post-VDZ.
- that patients with PSC/UC with ALP rises with VDZ would have a significant amount of $\alpha4\beta7$+ T-cells within their livers.

6.3 Methods

6.3.1 Patient cohort

I identified patients with PSC/IBD and patients with IBD alone who were prescribed VDZ for clinical purposes (usually for active IBD). Baseline demographics were collected. UCEIS and NI were determined from endoscopies and the associated biopsies at baseline and at some time-point post VDZ initiation (usually 4-6 months). A clinical response was defined endoscopically as UCEIS reduction from baseline of ≥2 (with baseline UCEIS ≥3), and histologically as NI reduction by ≥1 (with baseline NI ≥2). Endoscopic remission was defined as UCEIS ≤1 (with baseline ≥3) and histological remission was classified as Nancy ≤1 (with baseline NI ≥2).

6.3.2 Ethics

Patients recruited gave informed written consent to the study, "Inflammatory Bowel Disease in Oxford: prospective cohort for outcomes, treatment, and predictors" (REC Ref: 09/H1204/30). This project was an approved sub-project within this study, entitled, "Liver and intestine-specific lymphocytes in Primary Sclerosing Cholangitis and Inflammatory Bowel Disease". Patients who underwent fine needle aspiration of the liver (FNA liver) gave written informed consent to the study, "Analysis of liver infiltrating lymphocytes in primary sclerosing cholangitis by fine needle aspiration of the liver" (REC Ref: 17/SC/0290).

6.3.3 Sample collection

Peripheral blood samples were taken at baseline (at time of initial VDZ infusion, immediately before the infusion, or within 3 months prior to VDZ initiation) and at various time-points post-VDZ, usually immediately prior to VDZ infusion. Peripheral blood mononuclear cells (PBMC) were isolated and either frozen for future use or stained and used for flow cytometry.

Biopsies were taken from terminal ileum, inflamed colon and uninflamed colon where possible. Some patients had no uninflamed colon (pancolitis) and some patients had no inflammation (or minimal), so in these cases the relevant biopsies could not be taken. For a biopsy to be termed “uninflamed” patients must have had a UCEIS of 0 (i.e. an area where the UCEIS was 1, where there may be some erythema and/or loss of mucosal vascular pattern, would not be sampled as “uninflamed”). Similarly, for a sample to be taken as “inflamed colon”, patients must have a UCEIS of 2 or more, and the most inflamed areas were sampled. In some patients, the ileum could not be reached either due to technical reasons, or the patient was only having a flexible sigmoidoscopy, so on some occasions no ileal biopsy was taken. Lamina propria mononuclear cells (LPMC) were isolated from intestinal biopsies and stained fresh for flow cytometry as per above methods.

Liver infiltrating lymphocytes (LIL) were isolated from liver biopsies or FNA liver . They were stained fresh for flow cytometry analysis.

6.3.4 Flow cytometry analysis

Freshly stained PBMC, LPMC were stained with Panel 1 antibodies (cell surface staining) and acquired on the 3-laser LSR II flow cytometer (BD Biosciences, Berkshire, UK) either that day or within 3 days of staining and fixation. LIL were stained fresh with Panel 4, 5 or 6 antibodies (cell surface staining) and acquired on the 4-laser LSR II flow cytometer (BD Biosciences, Berkshire, UK).

The initial antibody panel used for staining (Panel 1) only examined CD4+ and CD8+ T-cell populations as a whole, and was used initially for analysis of PBMC and LPMC. It did not include CD45RA and hence could not differentiate naïve from memory T-cells. This was not an issue for LPMC as most lamina propria T-cells are memory T-cells. However, to ascertain differences among memory T-cell populations in PBMC, a new antibody panel (Panel 4) was developed containing CD45RA. Cryopreserved PBMC were thawed and stained with Panel 4, and acquired on the 4-laser LSR II flow cytometer either that day or within 3 days of staining and fixation. To detect $\alpha4\beta7$, only a $\beta7$ antibody was used, which was felt to be an appropriate surrogate, as the only other $\beta7$ integrin is $\alpha E\beta7$, which is on <3% of circulating T-cells, therefore the vast majority of $\beta7$+ cells would be $\alpha4\beta7$+.

6.4 Results

6.4.1 Peripheral blood changes with VDZ

6.4.1.1 Baseline demographics

Peripheral blood samples were available for 25 patients – 12 with PSC/IBD, 13 with IBD only. The baseline demographics are shown in *Table 6.1*. Of those with PSC/IBD, all

patients had UC. Two patients with PSC/UC were initially characterised as having IBD-unspecified (IBD-u), and one with CD, but all were subsequently reclassified as UC due to their clinical and endoscopic phenotype. Of those with IBD only, 12 had UC (1 re-classified from IBD-u) and 1 had CD. The patient with CD had an ileocolonic phenotype.

All patients had clinically active disease at time of VDZ commencement, as determined endoscopically, except for one patient with PSC/UC who was switched from an anti-TNF agent to VDZ due to an allergic reaction to the anti-TNF. As this patient's baseline UCEIS was 1/8, they were not included in the analyses for response/remission (and nor was the Crohn's patient).

Blood was taken from all 25 patients at baseline and up to three time points post-VDZ initiation ("Post-VDZ 1, 2, and 3", see *Table 6.2)*. Occasionally due to weekends, patient absence and scheduling conflicts, VDZ was given at time points a few days either side of the scheduled dose (e.g. 2nd dose given at day 16 instead of day 14, where the day the first dose of VDZ is given is day 0). Hence some of the time points do not fall on the exact VDZ schedule days (day 0, 14, 42, etc), but every endeavour was made for blood to be taken immediately prior to VDZ infusion, to ensure that the lowest concentration (trough) of VDZ was present in the peripheral blood at time of analysis.

Table 6.1 - Baseline demographics of patients with PSC/IBD and IBD only undergoing VDZ therapy (peripheral blood analysis)

Variable	PSC/IBD cohort (n=12)	IBD only cohort (n=13)	P-value
Age, mean (±SEM, range)	27.9 years (±2.5, 18.1-47.6)	45.6 years (±16.9, 22.5-70.8)	0.004
Male gender, n (%)	11 (91.7%)	7 (53.9%)	0.073
Type of PSC, n(%) Classical PSC Small Duct-PSC	 11 (91.7%) 1 (8.3%)	 0 (0%) 0 (0%)	n/a
Type of IBD Ulcerative Colitis Crohn's Disease	 12* (100%) 0 (0%)	 12* (92.3%) 1 (7.7%)	 >0.999 >0.999
Extent of disease (UC only)[#] E1 E2 E3	 0 (0%) 0 (0%) 12 (100%)	 0 (0%) 6 (50%) 6 (50%)	 >0.999 0.015 0.005
Immunomodulator at baseline	4 (33.3%)	5 (45.5%)	0.680
Steroids at baseline	3 (25.0%)	6 (46.2%)	0.411
5-ASA at baseline	7 (58.3%)	5 (38.5%)	0.434
UCEIS at baseline colonoscopy, mean (±SEM)	4.3 (±0.4)	4.5 (±0.2)	0.729
UDCA at baseline	7 (58.3%)	0 (0%)	0.002

*4 patients with UC were initially diagnosed at IBD-u (n=3) or CD (n=1), but re-classified as UC prior to VDZ initiation. [#]Montreal classification: E1=proctitis; E2=left-sided colitis; E3=extensive colitis.[218]
IBD, inflammatory bowel disease; n/a, not applicable; PSC, primary sclerosing cholangitis; SEM, standard error of the mean; UC, ulcerative colitis; UCEIS, ulcerative colitis endoscopic index of severity;[52] UDCA, ursodeoxycholic acid.

Table 6.2 - Time points of blood sample in relation to first vedolizumab dose.

Time point of blood sample	Number of patients with sample taken	Median (IQR, Range)
Pre-VDZ	25	-14 days (IQR -35 to 0, range -99 to 0)
Post-VDZ-1	25	Day 18 (IQR 14-75, range 12-161)
Post-VDZ-2	17	Day 151 (IQR 63-229, range 42-296)
Post-VDZ-3	3	Day 180 (IQR 95-291, range 95-291)

Note, first dose of VDZ was given on day 0, with negative numbers indicating the number of days prior to day 0.
IQR, interquartile ratio; VDZ, vedolizumab.

6.4.1.2 Effect of vedolizumab on CD4+ and CD8+ populations in peripheral blood

The proportion of CD4+ and CD8+ T-cells in the peripheral blood which were β7 positive declined significantly from baseline to various time-points post-VDZ (see *Figure 6-1*). At baseline, the median proportion of CD4+ T-cells which were β7 positive was 19.7% (IQR13.0-32.9), reducing to 11.0% (IQR6.6-16.7, p<0.0001) at post-VDZ-1 (median day 18), and further decreasing at later time points (post VDZ-2: median 9.1% [IQR7.9-12.3, p=0.0006]; post-VDZ-3: median 5.9% [IQR5.2-11.4, p=ns]). A similar decline was seen in the proportion of CD8+ T-cells expressing beta7+: baseline: median 26.1% (IQR13.6-41.5); post-VDZ-1: 12.7% (IQR8.0-21.3, p<0.0001); post-VDZ-2: 8.4% (IQR5.7-14.8, p<0.0001); post-VDZ-3: 9.3% (IQR6.8-10.9, p=ns).

As there was some variation in the duration from VDZ initiation at each time-point, I analysed the changes in a subset of patients for whom I had samples taken at set time-points: day 14, day 42, and day 120+. (allowing for a few days either side of these time points). The reduction in beta7 expression with VDZ remained statistically significant at each time point, and it was evident that beta7 expression was reduced as early as day 14, after a single dose of VDZ, and remained at similarly low levels throughout. Data for CD4+ cells at these time points are shown in *Figure 6-2*.

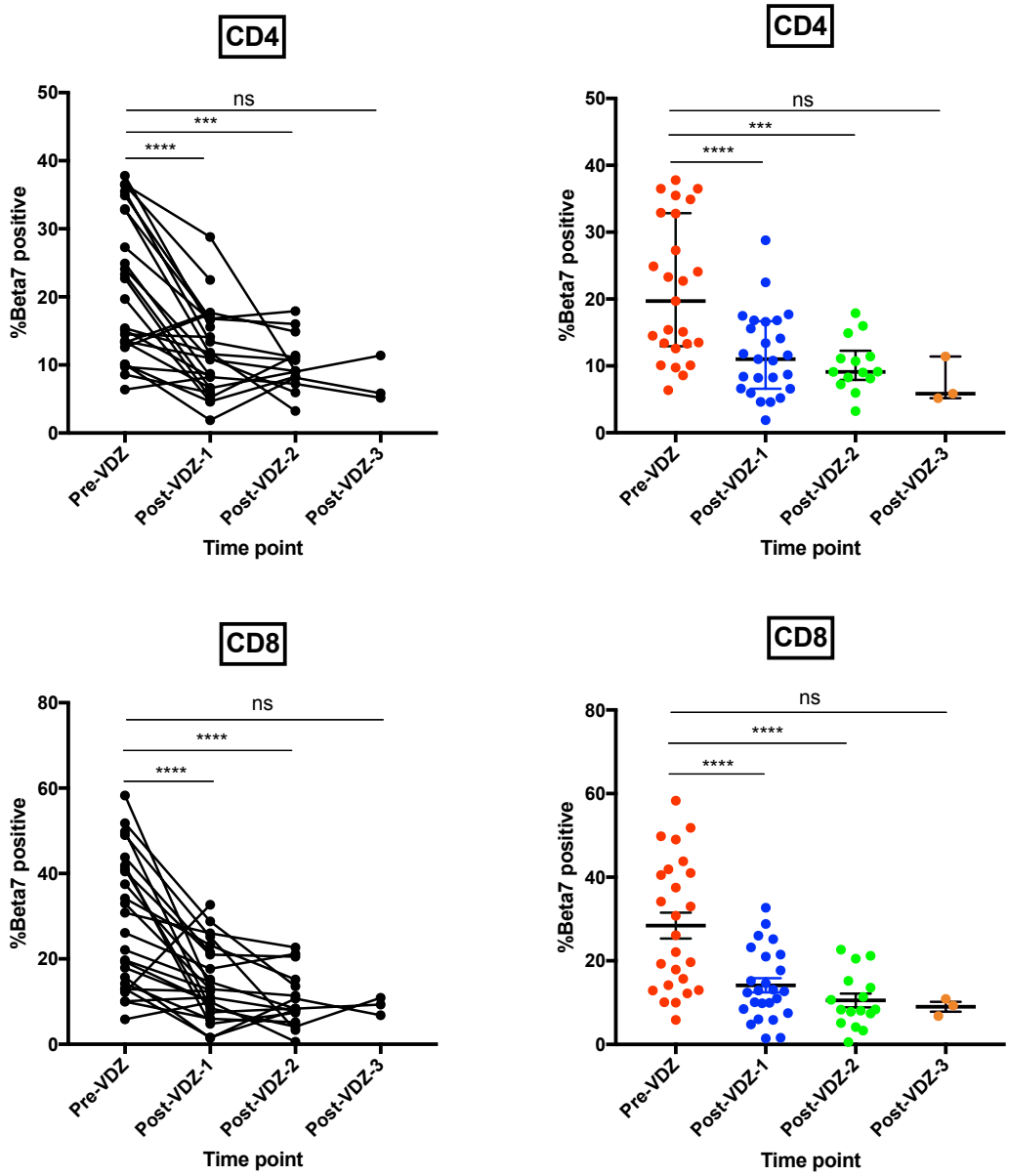


Figure 6-1 - The proportion of peripheral CD4+ and CD8+ T-cells which are β7 positive at various time points declines on vedolizumab.

Cell surface expression of β7 was analysed via flow cytometry on PBMC from blood samples of patients with either PSC/IBD or IBD alone, taken pre-VDZ (n=25), and at various time-points post-VDZ: post-VDZ-1 (n=25), post-VDZ-2 (n=14), and post-VDZ-3 (n=3). The duration from VDZ initiation to the subsequent time-points are shown in Table 6.2. Cell populations were gated upon CD4+ (top panels) and CD8+ (bottom panels), and the proportion of CD4+ and CD8+ T-cells which were β7+ was determined using a fluorescence minus one negative control. The left panel shows spaghetti plots depicting the course for each patient, and the right panels show the median β7 proportion (±SEM) among the relevant cell population. For the gating strategy used, please see Chapter 1. Paired T-test performed for parametrically distributed samples and Wilcoxon matched-pairs signed rank test performed for non-parametrically distributed samples. PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

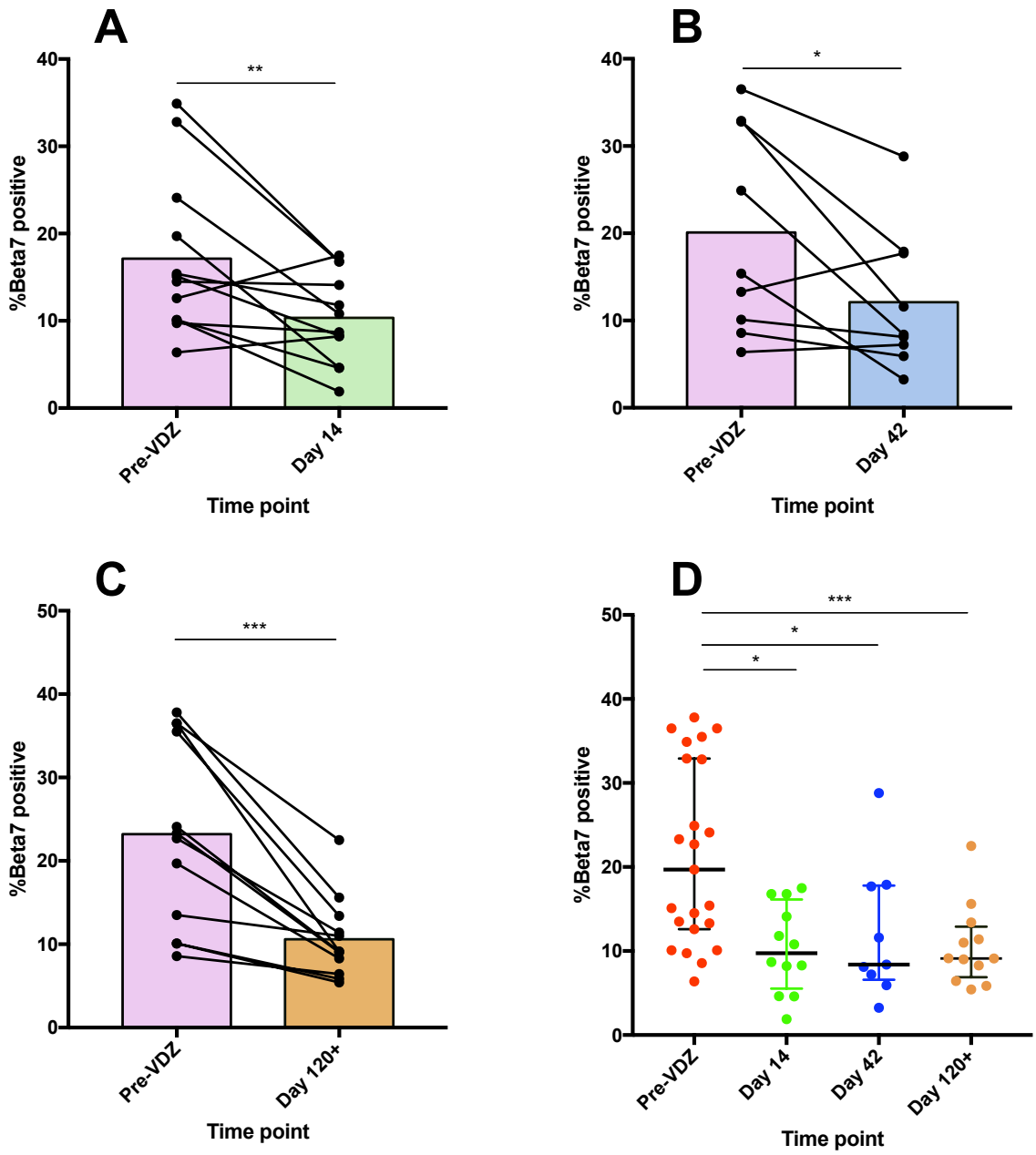


Figure 6-2 - Reduction of beta7 expression at particular time-points post-VDZ among peripheral CD4+ T-cells.

Cell surface expression of β7 was analysed by flow cytometry on PBMC from blood samples of patients with either PSC/IBC or IBD alone. The proportion of CD4+ T-cells which were β7+ was determined using a fluorescence minus one negative control. These proportions were compared between paired samples pre-VDZ and at a later time-point on VDZ: A, Day 14 (n=12), B, day 42 (n=9), and C, day 120+ (n=12). D shows all samples at these time-points, with median±IQR shown. Regardless of which time-point is chosen, the proportion of CD4+ T-cells which were β7+ declined with VDZ therapy to similar levels. Paired T-test performed for parametrically distributed samples (A and B) and Wilcoxon matched-pairs signed rank test performed for non-parametrically distributed samples (C, D). PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

When analysing the *per cell* expression of β7, with regards to comparing the median fluorescence intensity (MFI) at each time point, this also reduced after VDZ for both CD4+ and CD8+ T-cell populations, albeit only by small increments (see *Figure 6-3, A and B*). Among CD4+ T-cells, the mean MFI reduced from 350 at baseline (±SEM 32), to 288 at post-VDZ-1 (±21, p=0.016), to 271 at post-VDZ-2 (±85, p<0.0001). The mean MFI on CD8 T-cells at baseline was 625 (±94) and the drop at post-VDZ-1 was not statistically significant (541 ±51, p=ns), however, it dropped significantly to 488 at post-VDZ-2 (±47, p=0.009).

When looking at β7 expression on the β7+ populations themselves (as opposed to the whole CD4+ or CD8+ populations overall), the median MFI reduced among CD4+ β7+ T-cells from baseline to post-VDZ-1 (though not to statistical significance at post-VDZ-2), but did not significantly reduce among CD8+ β7+ populations (see *Figure 6-3, C and D*).

Due to the association between α4β7 and CCR9 and CD161 expression on T-cells, we also analysed the effect of VDZ on the expression of these cell surface receptors (see *Figure 6-4*). The proportion of peripheral CD4+ T-cells which expressed CCR9 gradually rose after treatment with VDZ (see). Whilst this was also true numerically for the CD8+ T-cell population, the rise did not reach statistical significance. There was no change in the proportion of CD4+ or CD8+ T-cells which co-expressed both CCR9 and β7 (data not shown), nor was there any change in CD161 expression on CD4+ or CD8+ T-cells with VDZ therapy.

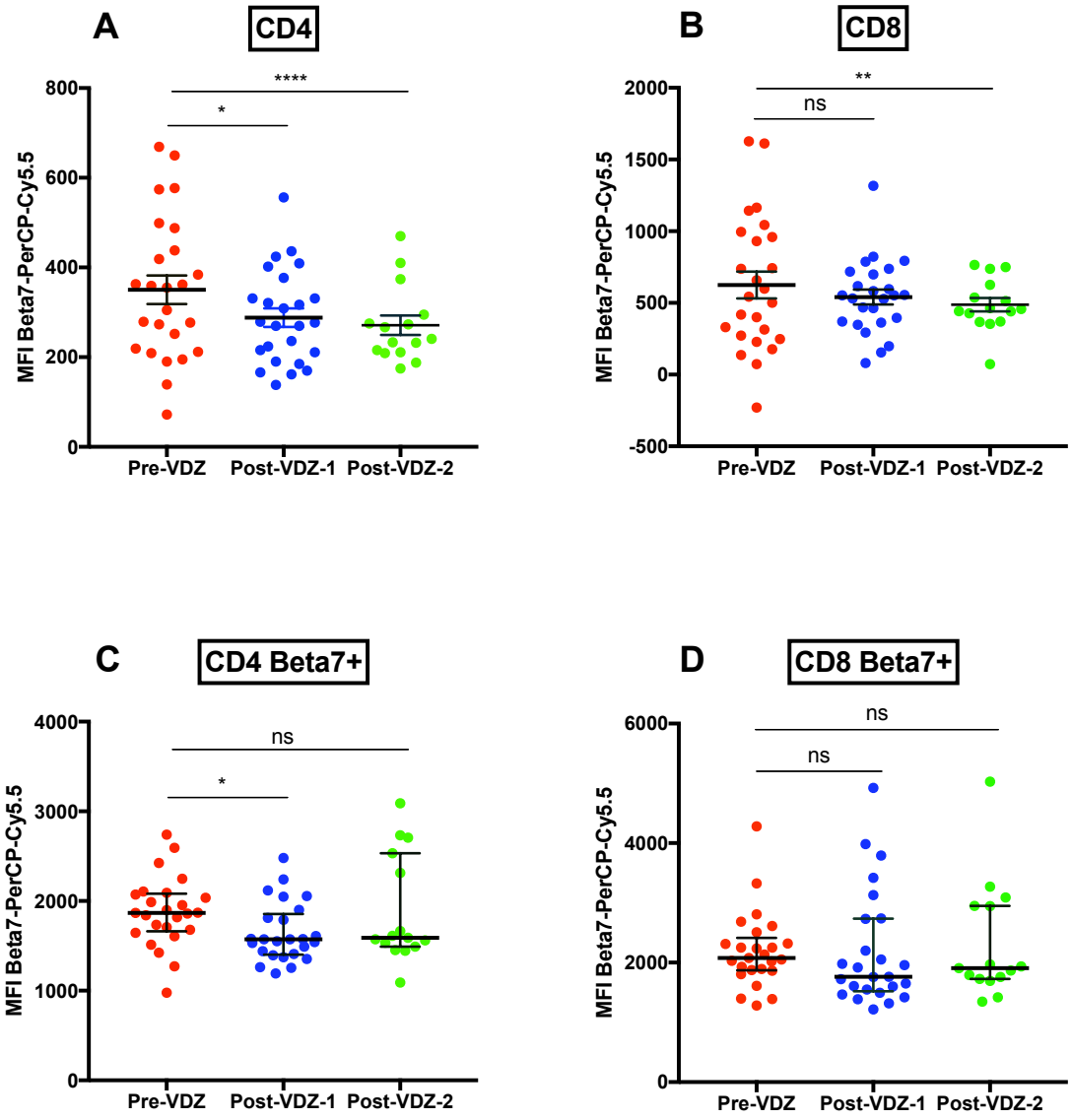


Figure 6-3 - Median fluorescence intensity of peripheral CD4+ and CD8+ T-cells reduces with vedolizumab.

Flow cytometry analysis was performed on PBMC from samples taken from patients with PSC/IBD or IBD alone, and the median fluorescence intensity of PE (which was conjugated to the β7 antibody) was determined for various cell populations. This was carried out prior to VDZ and at various time points after VDZ initiation. The cell populations depicted are: A,CD4+ T-cells; B, CD8+ T-cells; C, CD4+ β7+ T-cells; D, CD8+ β7+ T-cells. Mean±SEM are displayed in A and B, and median±IQR are shown in C and D. Paired T-test performed for parametrically distributed samples and Wilcoxon matched-pairs signed rank test performed for non-parametrically distributed samples. PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

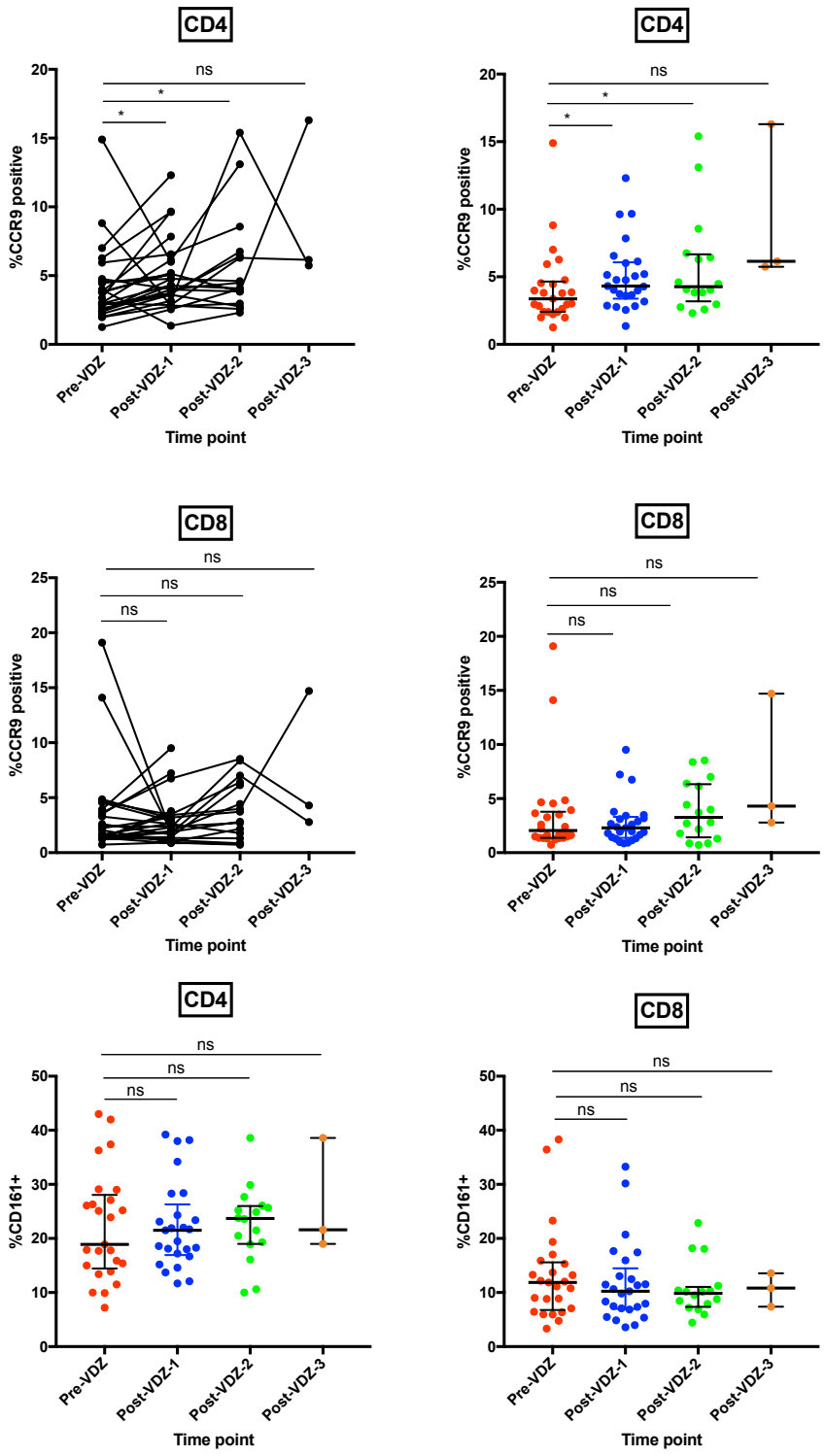


Figure 6-4 - CCR9 and CD161 expression on peripheral T-cells after therapy with vedolizumab.

Flow cytometric analysis of cell surface molecules among CD4 and CD8 T-cell populations pre-VDZ and at various time-points post-VDZ. Upper panel: The proportion of CD4+ T-cells expressing CCR9 increases with VDZ. Middle panel: There is no difference in the proportion of CD8+ T-cells which are CCR9+ with VDZ therapy. Bottom panel: The proportion of T-cells which are CD161+ does not change with VDZ; CD4+ T-cells represented on left, CD8+ T-cells represented on right. Median±SEM are shown. Paired T-test and Wilcoxon matched-pairs signed rank test applied as appropriate. VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

6.4.1.3 Differences between PSC/IBD and IBD alone with regards to the effects of VDZ on peripheral CD4+ and CD8+ T-cells

Patients with PSC/IBD had a greater drop in the proportion of both CD4+ and CD8+ T-cells which were β7+ with VDZ therapy compared with patients with IBD alone (see Table 6.3 and Figure 6-5). Patients with PSC/IBD had a more than 50% drop in the proportion of CD4+ T-cells which were β7+ (median 23.0% [IQR 13.0-34.4] at baseline to 9.5% [IQR 5.6-14.7, p=0.001] at post-VDZ-1 time-point, with further significant reductions at future time-points. Patients with IBD alone only dropped from a median 14.5% (IQR11.4-30.1) at baseline to 13.4% (IQR8.3-17.2, p=0.013) at post-VDZ-1 time-point, and no further significant drops at post-VDZ-2 time-point. Whilst at initial glance, it appears that the baseline proportion of CD4+ T-cells which are β7+ is higher in patients with PSC/IBD compared with IBD alone, this was not statistically significant.

There were no differences between PSC/IBD and IBD with regards to CCR9 proportionate changes among CD4+ and CD8+ T-cell populations with the exception of a small but statistically significant increase from baseline to post-VDZ-1 expression of CCR9 on CD4+ T-cells among patients with UC, where there was no change seen in PSC/IBD (data not shown). All other changes in CCR9 expression at further time-points were also not statistically significant, for both PSC/IBD and IBD cohorts.

Table 6.3 - PSC/IBD versus IBD patients - the proportion of CD4+ and CD8+ T-cells which were β7+ at baseline and with VDZ therapy

Cell population & patient cohort	Baseline	Post-VDZ-1		Post-VDZ-2		Post-VDZ-3	
	%β7+	%β7+	p-value	%β7+	p-value	%β7+	p-value
CD4+							
PSC/IBD	23.0% (13.9-34.4)	**9.5%** (5.6-14.7)	0.001	**9.1%** (7.9-10.9)	0.0008	5.9% (5.2-11.4)	0.211
IBD	14.5% (11.4-30.1)	**13.4%** (8.3-17.2)	0.013	8.8% (5.8-15.7)	0.076	n/a*	
CD8+							
PSC/IBD	37.4% (19.0-49.6)	**13.0%** (5.7-22.7)	0.0001	**9.5%** 6.6-14.0)	0.0006	9.3% (6.8-10.9)	0.165
IBD	19.3% (11.2-34.2)	**12.4%** (8.7-19.6)	0.074	8.1% (4.0-21.6)	0.055	n/a*	

The median value ±IQR is depicted. Comparisons were made between the post-VDZ value and the baseline value. Values which are statistically significant are written in red.
*n/a = not applicable, as no samples were taken from IBD patients at post-VDZ-3 time-point.
IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis; VDZ, vedolizumab.

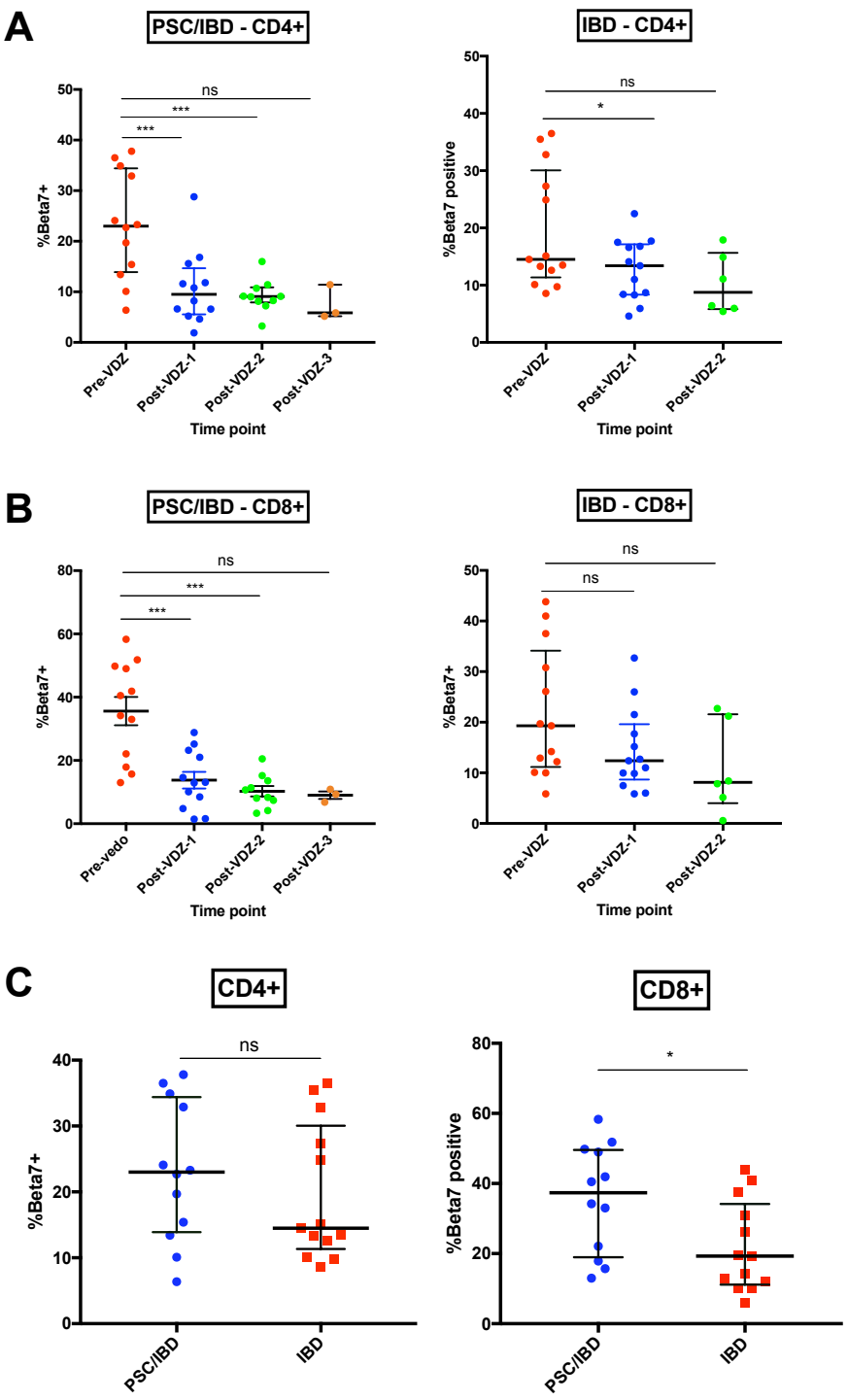


Figure 6-5 - PSC/IBD vs. IBD: Differences in β7 expression among peripheral CD4+ and CD8+ T-cell populations with vedolizumab therapy.

Flow cytometric analysis of the proportion of CD4+ and CD8+ T-cells which were β7+ at baseline and at various time-points post-VDZ. A, gated on CD4+ T-cells; B, gated on CD8+ T-cells. Patients with PSC/IBD (on left) had a greater drop in proportion of T-cells which were β7+ with VDZ therapy, compared with patients with IBD alone (right). C, baseline expression of β7 in PSC/IBD and IBD alone patients on CD4+ T-cells (left) and CD8+ T-cells (right). There was no difference in β7 expression between PSC/IBD and IBD on CD4+ T-cells, but there were more CD8+ β7+ T—cells in PSC/IBD compared with IBD alone. Graphs display median±IQR. Paired T-test and Wilcoxon matched-pairs signed rank test applied as appropriate. IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

6.4.1.4 Associations of β7+ on T-cells in the peripheral blood with endoscopic remission

Of the 25 patients included, 23 were able to be assessed for endoscopic remission for UC (11 PSC/UC, 12 UC). Two people were excluded: one for their diagnosis of Crohn's disease, and the second because their baseline UCEIS was only 1. Ten patients (43.5%) achieved an endoscopic remission - PSC/UC: 4/11 (36.4%) vs. UC: 6/12 (50%, p=0.680).

Both remitters and non-remitters had a decline with VDZ therapy of the proportion of CD4+ T-cells which were β7+ from baseline to post-VDZ-1 – only 3/25 patients had a rise in β7+ proportion (see *Figure 6-6*). There was no difference in the median change from baseline in remitters vs. non-remitters: -42.2% (IQR-60.7 to 5.1) versus -51.9% (IQR-65.5 to -22.2, p=0.522) respectively. These findings were similar among the CD8+ T-cell populations in remitters and non-remitters.

Remitters had a lower baseline proportion of β7+ T-cells compared with non-remitters – both among CD4+ and CD8+ T-cell populations. Furthermore, remitters had a lower proportion of CD8+ T-cells which were β7+ at post-VDZ-1 time-point (mean 9.6%±SEM1.99) as compared with non-remitters (17.7%±8.9, p=0.024), but this was not the case for the CD4+ population (remitters: 9.8%±1.6 vs. non-remitters: 14.1%±1.9, p=0.110). *Figure 6-7* shows these comparisons graphically.

There were no differences seen between remitters and non-remitters with regards to CCR9 expression (data not shown).

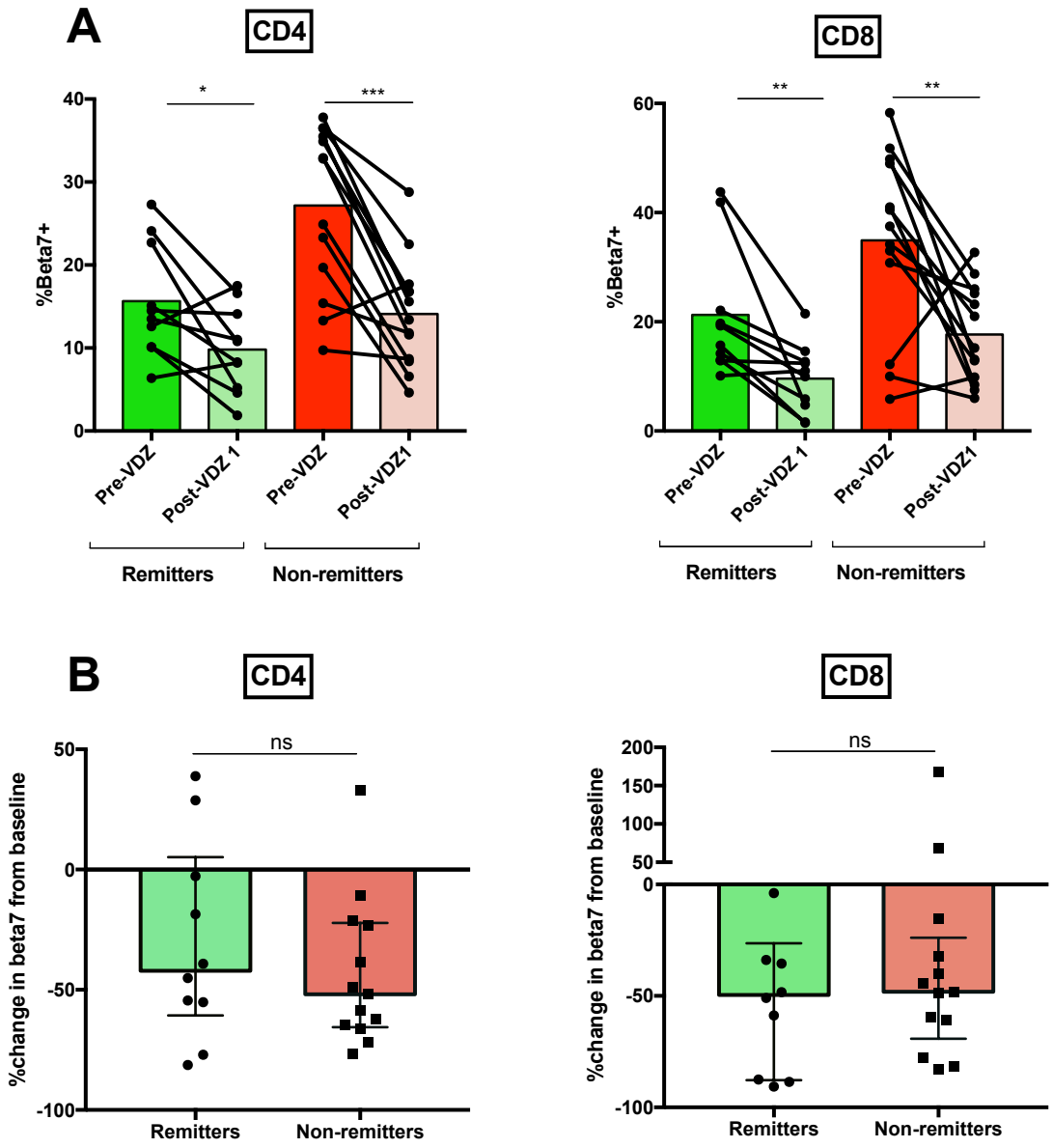


Figure 6-6 - Changes in proportions of β7+ CD4+ and CD8+ T-cells in remitters versus non-remitters on VDZ therapy.

Peripheral CD4+ and CD8+ T-cells were analysed via flow cytometry to ascertain the proportion of each cell population which was β7+ (using a fluorescence minus one negative control). Proportions of CD4+ and CD8+ T-cell populations which were β7+ were determined at baseline and at post-VDZ-1 time-point. A, spaghetti plots showing the change in proportion of β7+ cells in remitters (green) and non-remitters (red), as gated on CD4+ T-cells (left panel) and CD8+ T-cells (right panel). Bars represent the mean proportion, paired T-test performed for each group between baseline (pre-VDZ) and post-VDZ-1. B, the difference in the proportion of β7+ T-cells is shown as a percentage change from the baseline value among CD4+ T-cells (left panel) and CD8+ T-cells (right) in remitters versus non-remitters. Bars depict the median value, with IQR shown. Mann Whitney test performed. VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05

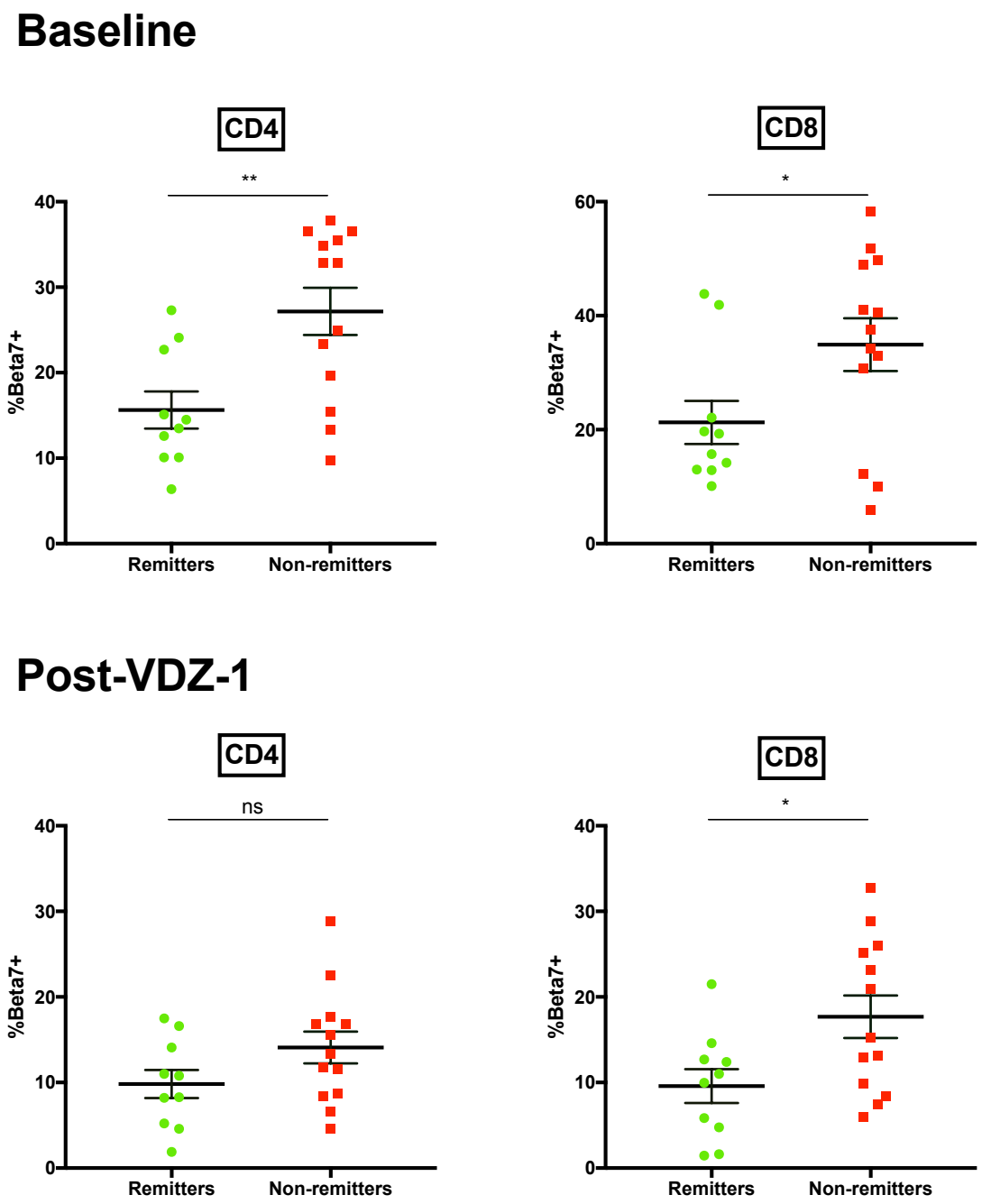


Figure 6-7 – Remitters have a lower proportion of peripheral blood T-cells which are β7+ at baseline and at follow-up compared with non-remitters.

The proportion of CD4+ (left panels) and CD8+ T-cells (right panels) which were β7+ were analysed via flow cytometry. The values were compared between remitters (green) and non-remitters (red) at baseline prior to VDZ (upper panels) and at post-VDZ-1 time-point (lower panels). Remitters, as compared with non-remitters, had lower β7 expression among CD4+ and CD8+ T-cells, as well as a lower proportion of CD8+ T-cells which were β7+ at follow-up on VDZ therapy. Mean±SEM is depicted. Unpaired t-test was performed. VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

6.4.1.5 Interim discussion on peripheral blood findings regarding CD4+ and CD8+ T-cell changes with VDZ therapy (Antibody panel 1)

These data show that upon therapy with vedolizumab, regardless of whether patients go into remission or not, the proportion of CD4+ and CD8+ T-cells which are β7+ decreases substantially. Furthermore, the expression per cell of the β7 receptor (as indicated by MFI) goes down in total CD4+ and CD8+ populations, but more so on CD4+ T-cells than CD8+ T-cells. Also, if one looks at the expression/MFI on the subset of β7+ T-cells, the β7 expression only decreases on CD4+ β7+ T-cells (not CD8+ β7+ T-cells). These findings could indicate that VDZ has more of an effect on T-cells which have a high expression of β7, such as CD4+ T-cells, and in particular, memory CD4+ T-cells, but I did not evaluate memory T-cells in this antibody panel. I therefore sought to evaluate this further on cryopreserved PBMC from the same cohort of patients, using a new antibody panel incorporating CD45RA to identify memory T-cells.

The finding that the proportion of CD4+ T-cells (but not CD8+ T-cells) which are CCR9+ increases with VDZ may reflect redistribution of the gut-homing CD4+ memory T-cells which have high expression of β7+ and are CCR9+. The reason we are seeing a *reduction* in the detection of β7, could be either that those cells are no longer there in the peripheral circulation, or, more likely, that the receptor is internalised on binding to VDZ, as had been shown *in vitro* in two previous studies.[144, 165] Another possibility is that the presence of VDZ in the peripheral blood, and its binding to α4β7 may prevent binding of the anti-human β7 monoclonal antibody we are using to detect these cells (Clone FIB504, Biolegend, London, UK). I therefore sought to test this hypothesis in a simple *in*

vitro experiment, co-culturing PBMC with varying concentrations of VDZ (or vehicle) prior to staining with the anti-human β7 monoclonal antibody for flow cytometric analysis.

Finally, the proportions of CD4+ and CD8+ T-cells which are β7+ are lower than what has been reported in the literature. The median proportion of CD4+ T-cells which were β7+ was 19.7% (IQR13-32.9), and for CD8+ T-cells was 26.1% (IQR 13.6-41.5). The literature suggests the proportion of CD4+ T-cells which are β7+ is 25-60%, and for CD8+ T-cells, 40-80%. The most likely explanation for this is that a fairly dim fluorochrome (PerCP-Cy5.5 was used on β7), and in hindsight, a more intense fluorochrome would have been more appropriate. Also, it is possible that the concentration used for the monoclonal antibody (1:25) may have been too dilute. Hence, in the next antibody panel, I used the same antibody but conjugated to a brighter fluorochrome and titrated its concentration within the panel to optimise its detection of β7.

6.4.1.6 Effects of *in vitro* culture of T-cells with vedolizumab on detection of cell surface β7 with flow cytometry

PBMC were isolated from 5 healthy human controls (over the course of 2 experiments) and cultured with varying concentrations of VDZ (0-200 μg/mL)for 2 hours at 37°C. The reason these concentrations of VDZ were chosen is because prior literature has suggested peripheral blood trough concentrations of patients on VDZ therapy reach levels as high as 142μg/mL.[219, 220] However, VDZ concentration levels vary and are more commonly in the range of 10-35μg/mL. As I was attempting to mimic the environment within the peripheral blood from which these samples were taken, I tested 5 concentrations of VDZ from 0 to 200μg/mL.

The cells subsequently underwent cell surface staining with an antibody panel (Panel 4) containing the same clone of monoclonal antibody against human β7 used in the prior experiments, but conjugated to a different fluorochrome (phycoerythrin, PE). This panel had previously undergone dose titration with healthy control PBMC to optimise the intensity of each antibody.

β7 expression was determined on CD4+ and CD8+ T-cells, as well as on their memory subpopulations (CD45RA-), via multicolour flow cytometry (see *Figure 6-8* for gating strategy). The proportions which were β7+ were compared among differing concentrations of VDZ exposure.

There was no difference in β7 expression across differing levels of exposure of VDZ among both CD4+ and CD8+ T-cells as determined both by proportions of these populations which were β7+ and by MFI (see *Figure 6-9*). Furthermore, the mean proportion of CD4+ and CD8+ T-cells which were β7+ was higher than those quoted in the prior results using antibody panel 1. In this case, the mean proportion of CD4+ T-cells which were β7+ (among the vehicle control group) was 51.7% (±SEM 3.1), and for CD8+ T-cells was 62.0% (±8.6). The expression of β7 did not vary among the differing VDZ exposures for the CD4 and CD8 memory cell populations either (data not shown).

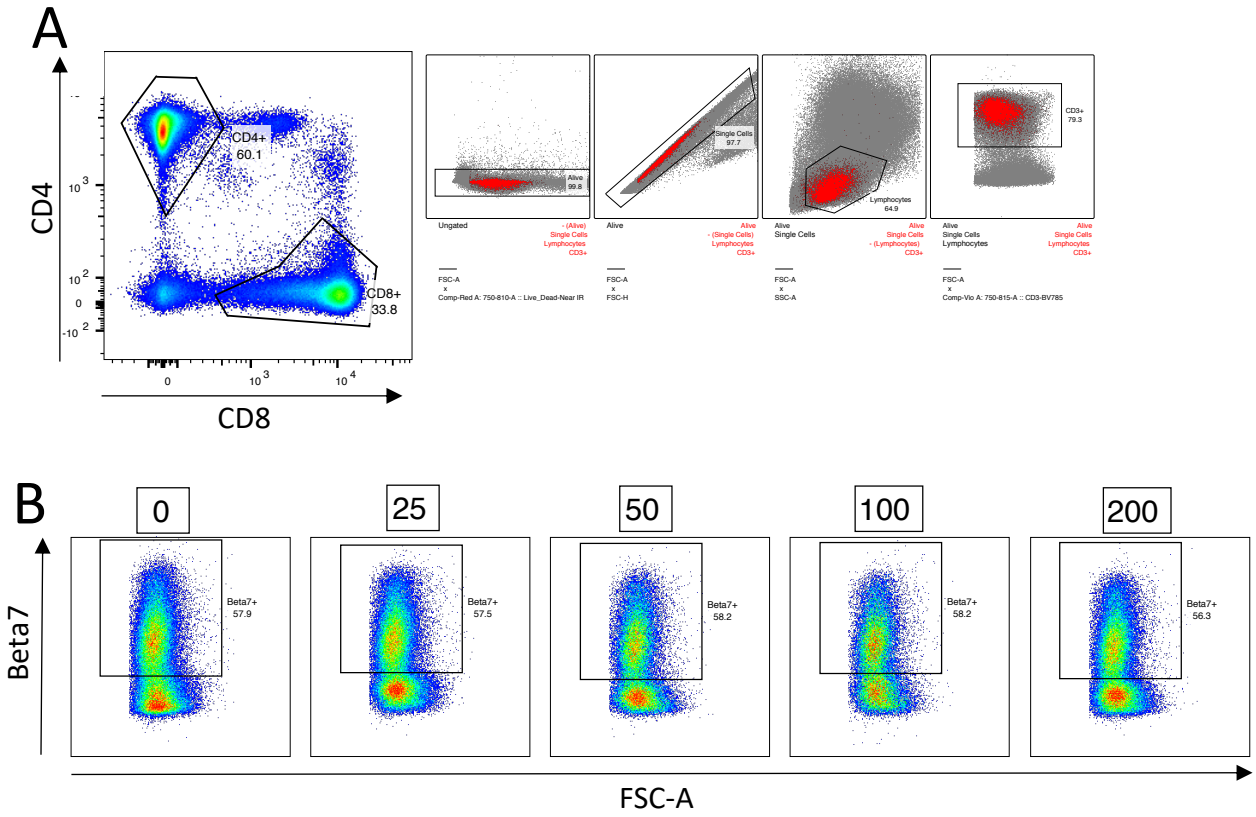
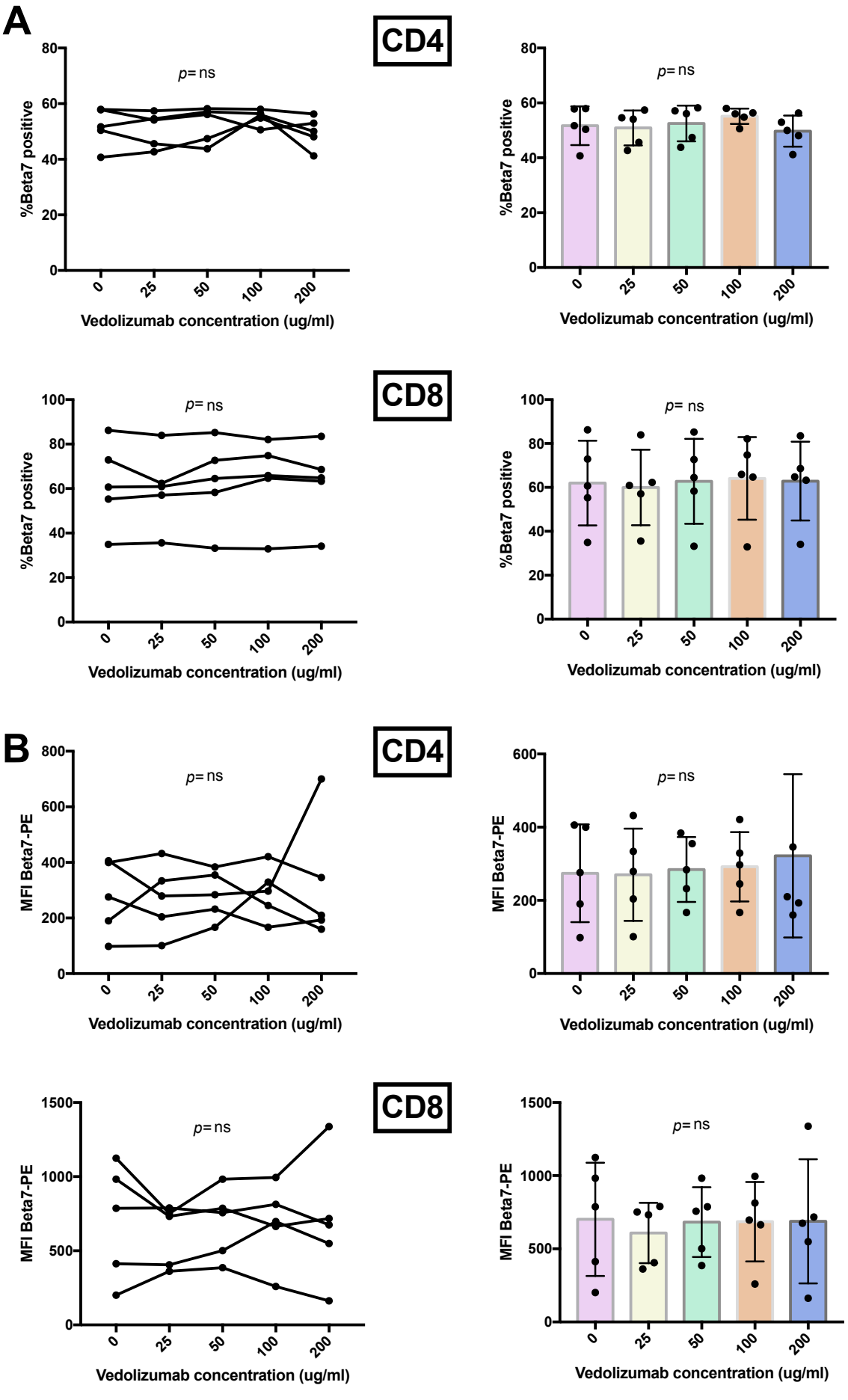


Figure 6-8 – (left) Gating strategy for β7+ T-cell populations for vedolizumab culture experiment

PBMC were incubated with varying concentrations of VDZ for 2 hours before undergoing cell surface staining and flow cytometric analysis. A, representative scatter plot showing identification of CD4+ and CD8+ populations and back-gating. B, representative scatter plot of a single patient's CD4+ T-cells which have undergone differing concentrations of VDZ exposure (numbers above each plot indicate concentration in μg/mL), showing gating for β7+ cells. C, Representative histogram (gated on CD4+ T-cells) showing β7 expression on the same patient for 5 different VDZ concentration exposures. FSC-A, forward scatter-area; PBMC, peripheral blood mononuclear cells.

Figure 6-9 – (next page) Expression of β7 on peripheral CD4+ and CD8+ T-cells with exposure to various in vitro concentrations of VDZ.

PBMC from 5 healthy controls were cultured in vitro with 5 different concentrations of VDZ (including 0μg/mL), and subsequently underwent cell surface staining with an antibody panel including anti-human β7 monoclonal antibody. β7+ cells were identified by multicolour flow cytometry. A, The proportion of CD4+ T-cells (upper panel) and CD8+ T-cells (lower panel) which were β7+ did not vary with differing VDZ concentrations. B, The MFI of β7-PE did not vary among differing VDZ concentrations. For all graphs, left panels show each individual patient, right panels show mean±SEM. One way ANOVA performed. MFI, median fluorescence intensity; ns, not significant; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; VDZ, vedolizumab.


A
CD4
p= ns
%Beta7 positive
Vedolizumab concentration (ug/ml)
CD8
B
MFI Beta7-PE

These data indicate that the presence of bound VDZ on CD4+ and CD8+ T-cells does not affect the binding of β7 antigen using the anti-human β7 monoclonal antibody (clone FIB504), presumably due to a different epitope being targeted. Therefore, the reduction of β7+ CD4+ and CD8+ T-cells in the peripheral circulation with VDZ therapy is likely to be a true finding. Furthermore, conjugation of this antibody to a more intense fluorochrome, such as PE, allows improved detection of β7 antigen, with values more consistent with those reported in the literature.

6.4.1.7 Issues with flow cytometric staining of thawed cells

This antibody panel (Panel 4) was utilised to stain cryopreserved PBMC from the patients described earlier (pre- and post-VDZ). The aims were to reproduce the findings on CD4+ and CD8+ populations with regards to β7 expression, but using the more intense fluorochrome of PE conjugated to the β7 antibody; to re-analyse CCR9 expression using the same monoclonal anti-human CCR9 antibody, but at a stronger (optimised) concentration; and to evaluate the effect of VDZ on these markers within the memory T-cell populations.

The forward versus side-scatter plots for these thawed PBMC had a different appearance to those of fresh PBMC (see *Figure* 6-10). The lymphocyte population was demarcated into two populations (after sorting on alive cells and singlets). The population which consisted of smaller cells (as indicated by a lower fluorescence on the forward scatter area (FSC-A) detector) were not present on the freshly stained PBMC. Furthermore, this population was significantly different from the other lymphocyte population, in that it has a vastly higher CCR9-APC expression.

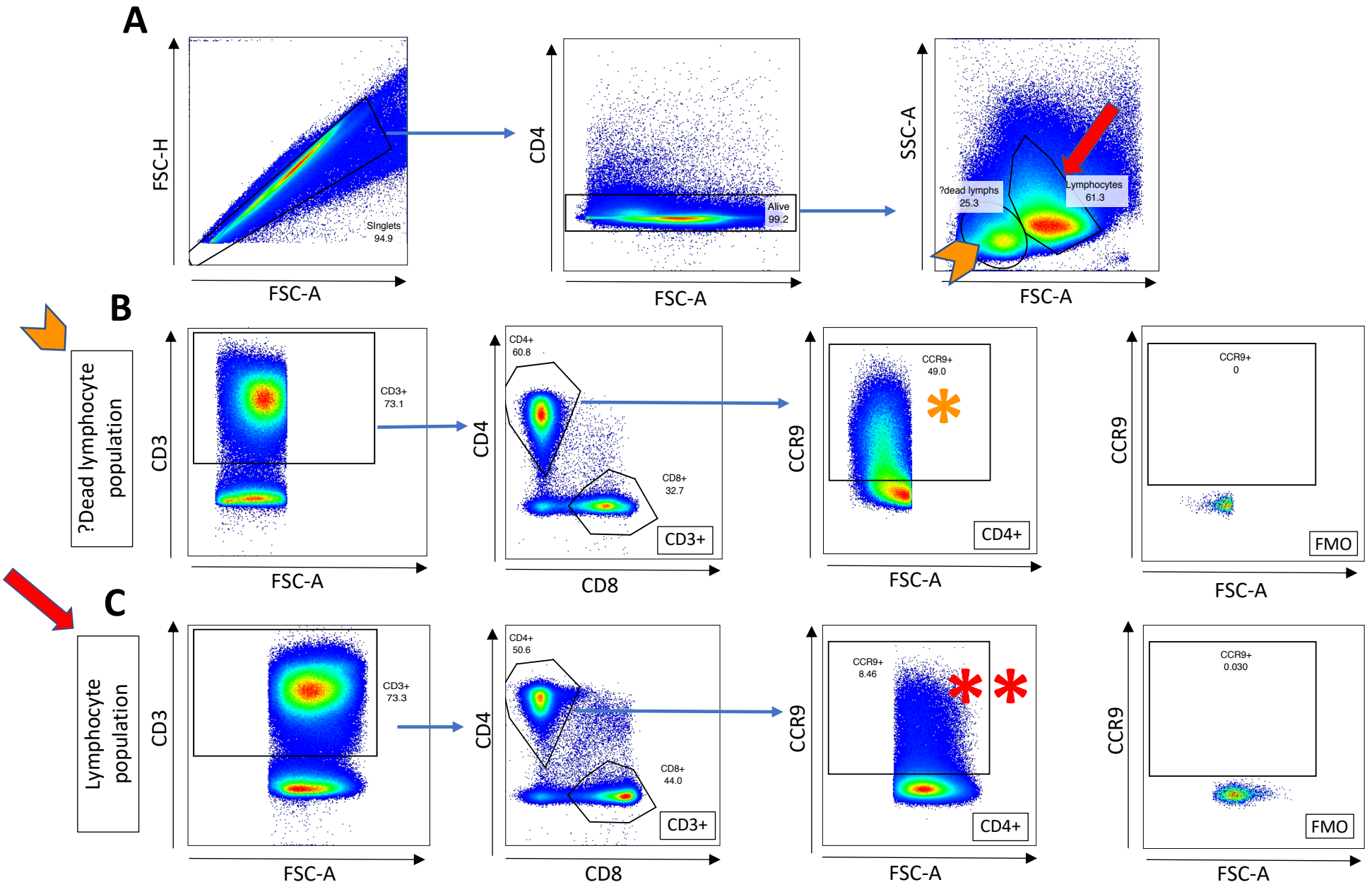


Figure 6-10 - Flow cytometry analysis of thawed peripheral blood mononuclear cells and the effect of differing viability dyes.

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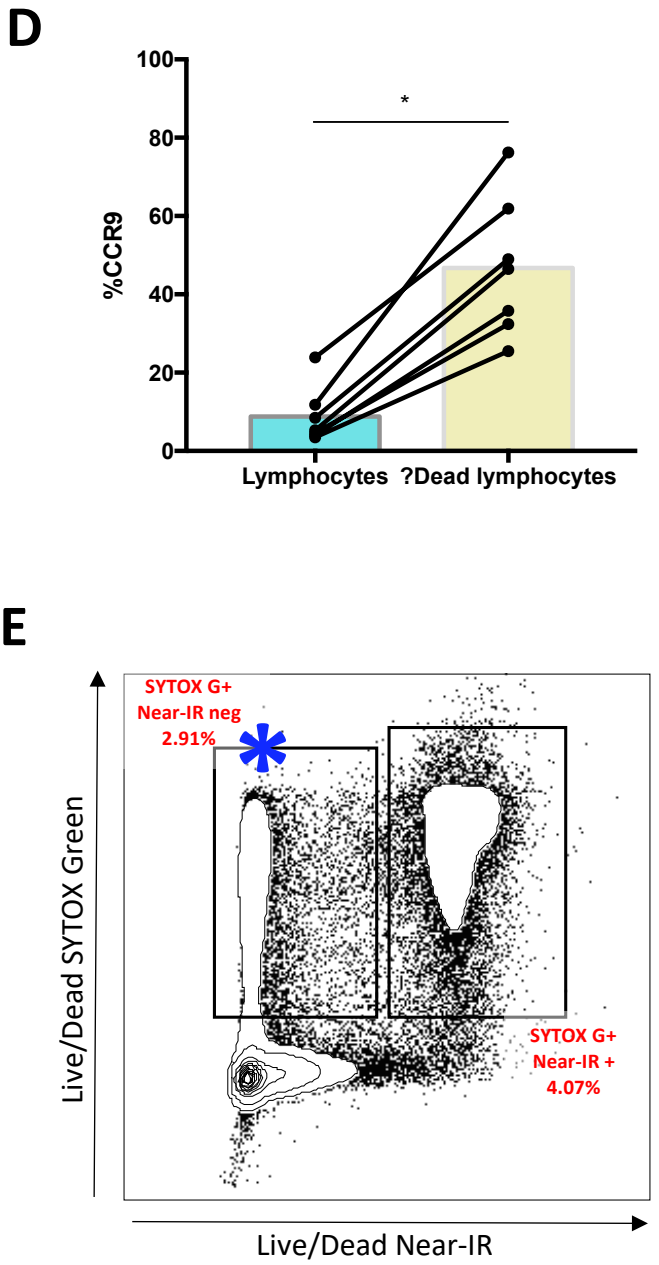


Figure 6-11 – (above and previous page) Flow cytometry analysis of thawed peripheral blood mononuclear cells and the effect of differing viability dyes.

Thawed PBMC were stained with antibody Panel 4. A, gating strategy yielded two distinct lymphocyte populations – a smaller population suspected to be dead or dying cells (orange chevron) and a larger population thought to represent the viable lymphocytes (red arrow). B and C, gating strategy following on from A to identify CCR9+ CD4+ T-cells. B represents the suspected dead population and C represents the suspected viable lymphocyte population. The suspected dead population has a very high proportion of CD4+ cells identified as CCR9-APC positive (*) compared with the suspected viable lymphocyte population (**). The respective FMOs are shown. C, the proportion of CD4+ T-cells which are CCR9+ (on a subset of thawed cells) according to the two populations. E, representative scatter plot of thawed PBMC (gated on singlets) stained with two differing viability dyes: SYTOX green dead cell stain and Near-IR fixable amine-reactive dead cell stain. Around 40% of dead cells were detected by SYTOX green but not Near-IR (*). FSC-A, forward scatter-area; FSC-H, forward scatter-height; IR, infrared; PBMC, peripheral blood mononuclear cells; SSC-A, side scatter-area.

It is known that thawed cells have a higher rate of cell death compared with freshly isolated cells. Therefore a viability marker is used to exclude non-viable or dead cells. It is also known that dead cells have a propensity to bind to monoclonal antibody fluorochrome conjugates (i.e. non-specific binding). In this experiment, the live/dead fixable near-infrared (IR) dead cell stain kit (ThermoFisher Scientific, Loughborough, UK) was used to determine viability of cells prior to fixation. This kit uses an amine reactive dye which reacts with free amines both in the cell interior and on the cell surface to detect dead cells.

It was suspected that this dye may not be detecting all of the dead cells in the thawed cell suspension, and a separate experiment was carried out within the laboratory by a colleague, Lucy Garner. Thawed PBMC were stained with both the near-IR amine reactive viability dye and a second viability dye – SYTOX green dead cell stain (ThermoFisher Scientific, Loughborough, UK) which binds to double-stranded DNA. Approximately 40% of dead cells (as detected by SYTOX green positivity) were not positive for near-IR, indicating that the amine-reactive dye did not detect all dead cells, or perhaps dying cells (see *Figure 6-10-E*).

Thus, for gating on lymphocytes in the experiment of thawed PBMC utilising antibody panel 4, it was assumed that the smaller lymphocyte population with the high CCR9 expression were in fact dead or dying cells, and not viable lymphocytes. The high CCR9 expression likely represented non-specific binding of the APC (which was conjugated to the CCR9 monoclonal antibody) to dead cells. For the results described below, only the larger lymphocyte population (as indicated by the red arrow in *Figure 6-10-A*) was used

for analysis, with suspected dead cells (as indicated by orange chevron) not included within the lymphocyte gate.

6.4.1.8 Effect of vedolizumab on peripheral blood memory T-cell populations

Of the 25 patients treated with VDZ, thawed PBMC were available for 19 patients pre-VDZ, 20 patients at post-VDZ-1 time-point, and 10 patients at post-VDZ-2 time-point. Paired samples from pre-VDZ to post-VDZ-1 were available for 17 patients (PSC/IBD n=7, IBD n=10).

Using this panel of antibodies, containing the β7 monoclonal antibody conjugated to the more intense fluorochrome, PE, analysis confirmed that the proportion of CD4+ and CD8+ T-cells which are β7+ decreases with VDZ (see *Figure 6-12*). The mean values were roughly double those found using antibody panel 1 which used the less intense fluorochrome, PerCP-Cy5.5, conjugated to the β7 monoclonal antibody.

However, when analysing memory T-cell populations, there is no change in β7 expression among CD4+ CD45RA- T-cells pre- versus post-VDZ, though there is still a small reduction among CD8+ CD45RA- T-cells. Although there is no change in the proportion of cells expressing β7 among CD4+ memory cells, the change in the distribution of the fluorescence intensity is striking. Intense expression of β7 is lost post-VDZ on CD4+ CD45RA- T-cells as shown in *Figure 6-13*. This correlates with a significant reduction of MFI in β7+ CD4+ memory T-cells, which also occurs among β7+ CD8+ memory T-cells. Hence, among CD4+ memory T-cells, the proportion of β7+ cells does not change, but the expression of β7 reduces on this β7+ population.

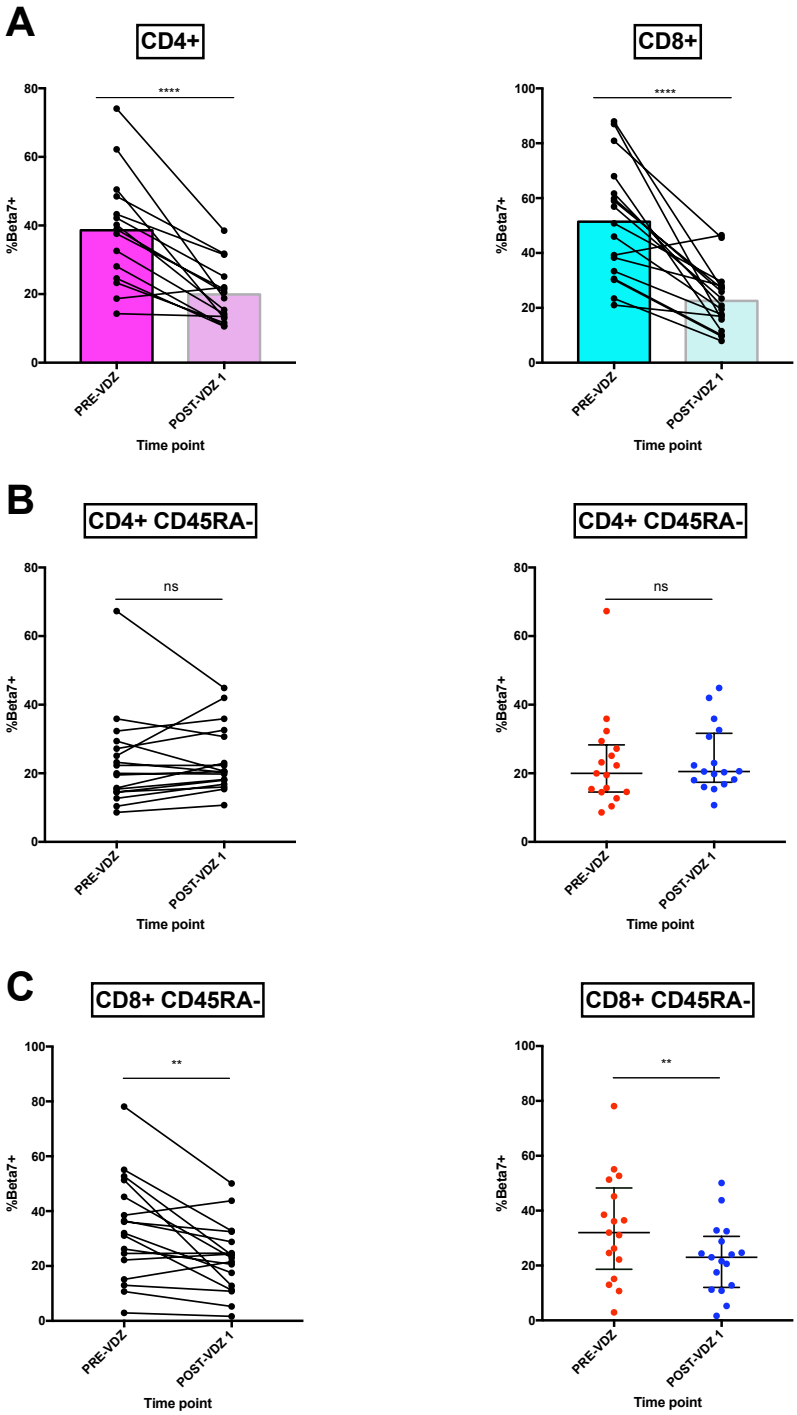


Figure 6-12 - β7 expression on cryopreserved peripheral CD4+ and CD8+ T-cells, and their memory counterparts, pre- and post-vedolizumab.

Cryopreserved PBMC were thawed and stained with antibody panel 4 before data acquisition with flow cytometry . A, the proportion of CD4+ T-cells (left) and CD8+ T-cells (right) which were β7+ reduced post-VDZ. Bars represent the mean value, paired T-test performed. B, The proportion of CD4+ 45RA- memory T-cells which were β7+ did not decrease with VDZ. Wilcoxon matched-pairs signed rank test performed, median±IQR depicted. C, there was a small but statistically significant decrease in the proportion of CD8+ CD45RA- memory T-cells which were β7+. Paired T-test performed, median±IQR depicted. PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

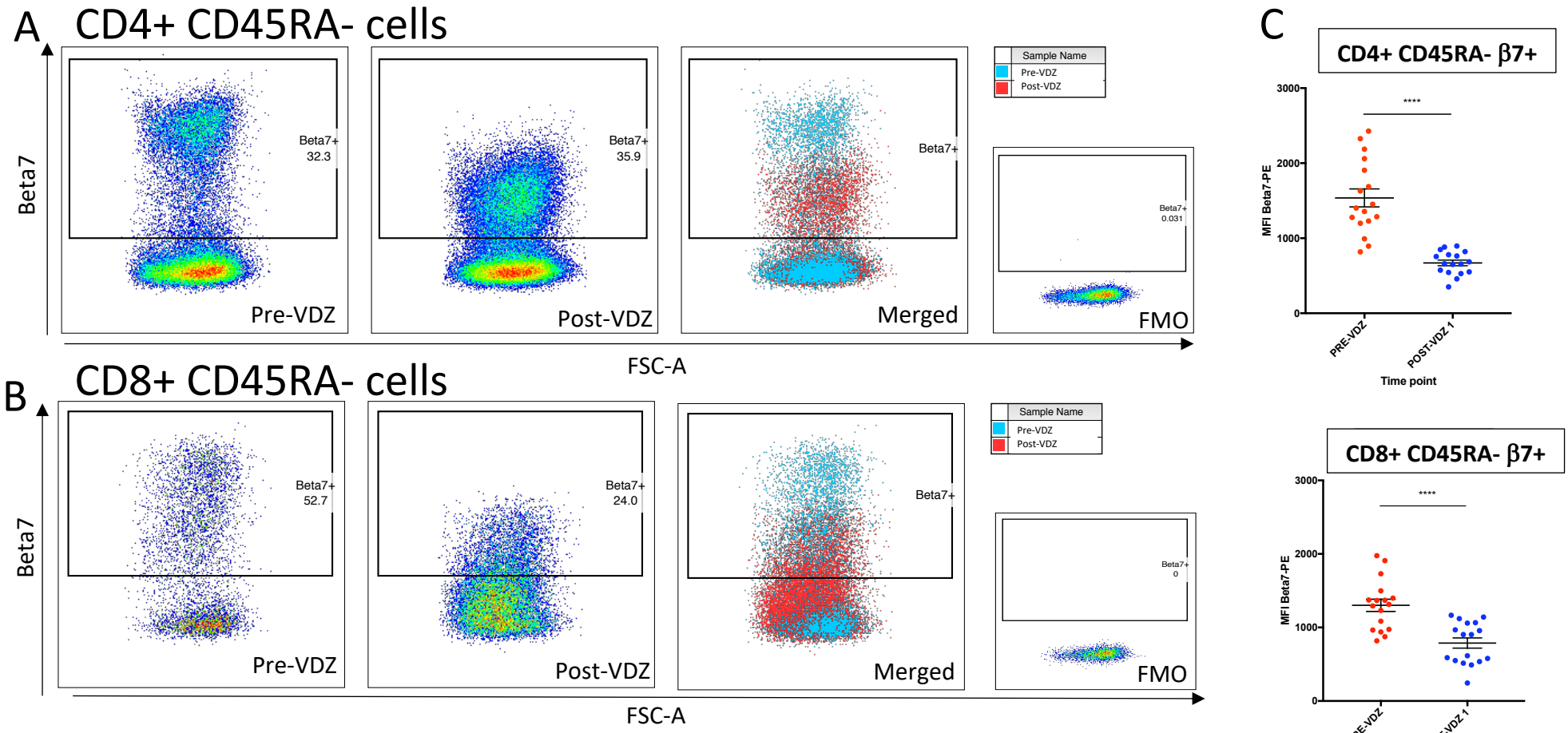


Figure 6-13 - β7 fluorescence intensity on CD4+ and CD8+ memory T-cells before and after vedolizumab.

Flow cytometric analysis was performed on thawed PBMC taken from patients pre- and post-VDZ. A and B, representative scatter plots of β7 expression on A, CD4+ CD45RA- T-cells and B, CD8+ CD45RA- T-cells. The left image shows pre-VDZ, the second image shows post-VDZ, and the third image shows the two images overlaid (blue: pre-VDZ, red: post-VDZ). FMO negative controls for respective cell populations shown on right. C, Mean MFI (±SEM) of β7-PE on CD4+ CD45RA- T-cells (upper graph) and CD8+ CD45RA- T-cells (lower graph). Paired t-test performed. FMO, fluorescence minus one; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

With regards to CCR9 expression, both the proportion of CD4+ and CD8+ memory T-cells which were CCR9+ increased with vedolizumab, and to a greater degree among the former (See *Figure 6-14*). Similarly, the proportion of CD4+ memory T-cells which were CCR9+/Beta7+ (dual positive) increased with VDZ, though the proportion stayed the same among CD8+ memory T-cells.

6.4.1.9 Differences between PSC/IBD and IBD alone with regards to the effects of VDZ on peripheral CD4+ and CD8+ T-cells

Unlike in CD4+ and CD8+ total populations, where patients with PSC/IBD had a greater drop in β7 expression with VDZ compared with IBD alone patients, there was no difference in β7 expression among memory T-cell populations between PSC/IBD and IBD patients. This is with the exception of a small but statistically significant drop in the proportion of CD8+ CD45RA- T-cells which were β7+ among IBD alone patients, which did not occur for patients with PSC/IBD. However, the numbers for paired analysis in these two cohorts were small (PSC/IBD n=7, IBD n=10), so this difference must be interpreted with caution.

However, only patients with PSC/IBD had a rise in CCR9 expression among CD4+ and CD8+ memory T-cells with VDZ, and not patients with IBD alone. The same was true for dual expression of CCR9/β7 – this only rose among memory T-cell populations in patients with PSC/IBD and not IBD alone.

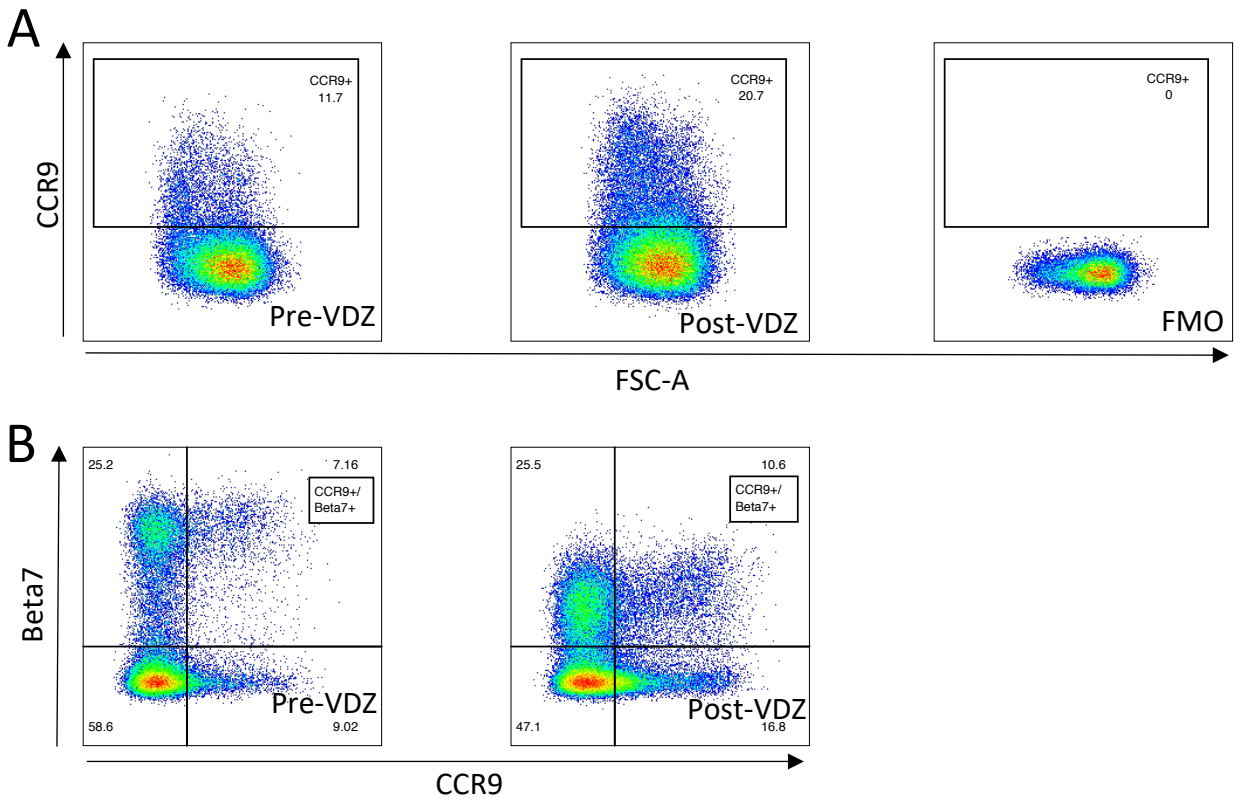
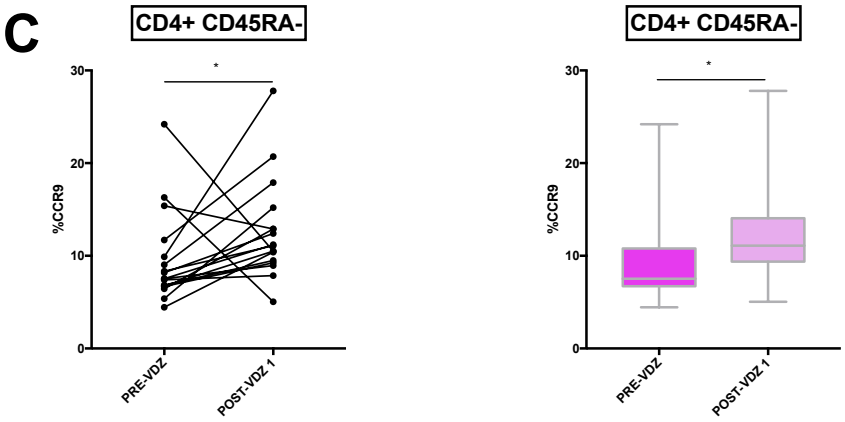
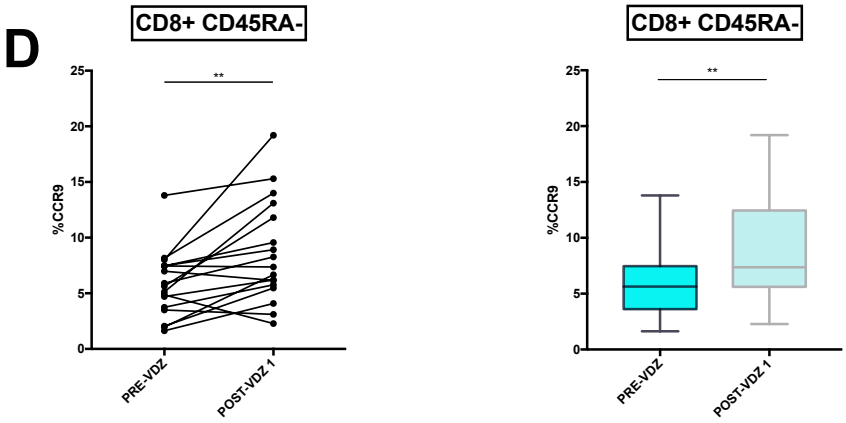
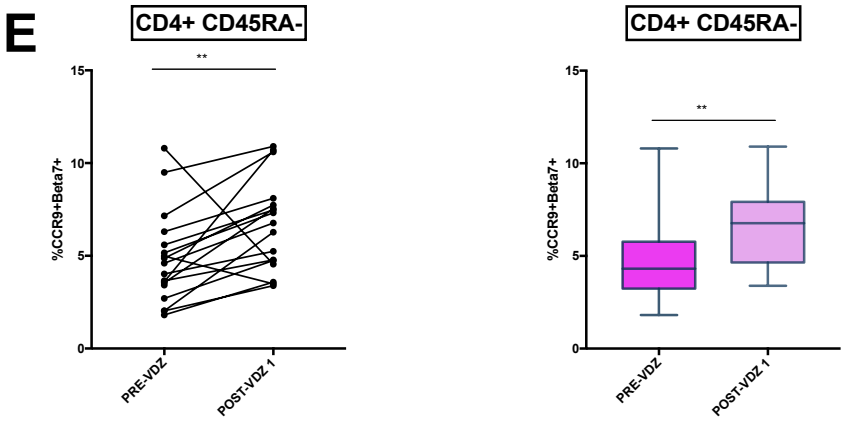


Figure 6-14 – (Above and next page) Flow cytometry analysis of CD4+ memory T-cells - CCR9 expression, and CCR9/β7 co-expression after vedolizumab therapy.

Cryopreserved PBMC from PSC/IBD and IBD patients pre- and post-VDZ were thawed and stained for flow cytometry analysis. A and B, representative scatter plots gated on CD4+ CD45RA- T-cells showing the proportion of cells which are A, CCR9+ and B, CCR9+/Beta7+ (dual positive). The left plots are from PBMC taken pre-VDZ, and the middle plots are the same patient post-VDZ. The FMO negative control is shown on the upper right. C and D, Paired analysis of PBMC pre- and post-VDZ shows increasing proportions of CCR9+ cells among CD4+ CD45RA-T-cells (C) and CD8+ CD45RA- T-cells (D). E and F, similarly, the proportion of cells which are CCR9+/β7+ (dual positive) increases post-VDZ among CD4+ CD45RA- T-cells (E) but not CD8+ CD45RA- T-cells. C-F, left graphs show each individual patient trajectories, right graphs show median±IQR as depicted by the box and minimum to maximum as depicted by the whiskers. Wilcoxon matched-pairs signed rank test and paired t-test carried out as appropriate. FMO, fluorescence minus one; FSC-A, forward scatter-area; PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.


C
CD4+ CD45RA-
%CCR9
PRE-VDZ
POST-VDZ 1
*
CD4+ CD45RA-
%CCR9
PRE-VDZ
POST-VDZ 1
*


D
CD8+ CD45RA-
%CCR9
PRE-VDZ
POST-VDZ 1
**
CD8+ CD45RA-
%CCR9
PRE-VDZ
POST-VDZ 1
**


E
CD4+ CD45RA-
%CCR9+Beta7+
PRE-VDZ
POST-VDZ 1
**
CD4+ CD45RA-
%CCR9+Beta7+
PRE-VDZ
POST-VDZ 1
**

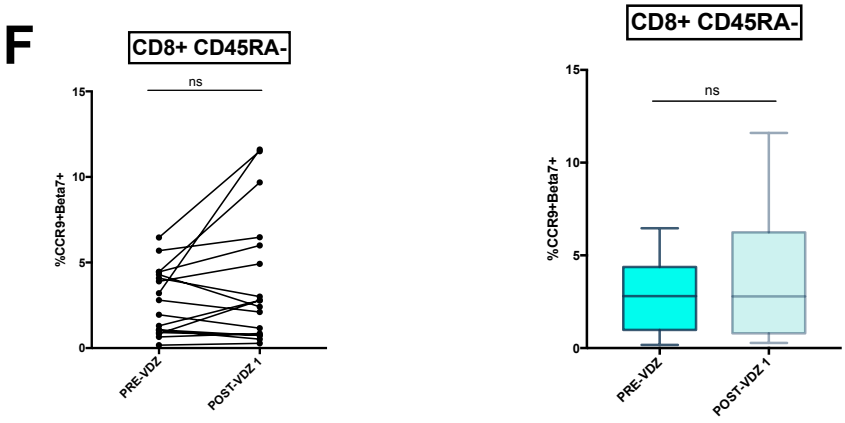

F
CD8+ CD45RA-
%CCR9+Beta7+
PRE-VDZ
POST-VDZ 1
ns
CD8+ CD45RA-
%CCR9+Beta7+
PRE-VDZ
POST-VDZ 1
ns

Table 6.4 - Table 1.6 - PSC/IBD versus IBD patients - the proportion of CD4+ and CD8+ memory T-cells which were β7+ or CCR9+ at baseline and with VDZ therapy.

Cell population & patient cohort	Baseline	Post-VDZ-1	
	%β7+	%β7+	p-value
CD4+ CD45RA-			
All patients	20% (14.6-28.3)	20.5% (17.4-31.7)	Ns
PSC/IBD	27.2% (15.4-32.3)	32.6% (18.2-42.0)	0.813
IBD	17.6% (12.1-22.5)	20.0 (16.5-22.5	0.247
CD8+ CD45RA-			
All patients	32.0% (18.7-48.3)	**23.0% (12.0-30.7)**	**0.004**
PSC/IBD	38.5% (24.6-52.7)	24.7% (21.6-43,=.8)	0.129
IBD	28.7% (12.4-38.7)	**19.1% (9.4-25.5)**	**0.008**
Cell population & patient cohort	**Baseline**	**Post-VDZ-1**	
	%CCR9+	**%CCR9+**	**p-value**
CD4+ CD45RA-			
All patients	7.5% (6.7-10.80	**11.1% (9.4-14.1)**	**0.016**
PSC/IBD	6.8% (6.5-8.3)	**10.4% (9.3-12.9)**	**0.016**
IBD	8.6% (7.2-15.6)	11.8% (9.8-15.9)	0.432
CD8+ CD45RA-			
All patients	5.6% (3.6-7.5)	**7.4% (5.6-12.5)**	**0.003**
PSC/IBD	5.9% (3.5-8.0)	**8.9% (6.7-14.0)**	**0.021**
IBD	5.0% (3.3-7.5)	6.2% (5.1-10.5)	0.106

The median value ±IQR is depicted. Comparisons were made between the post-VDZ value and the baseline value. Values which are statistically significant are written in red.
IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis; VDZ, vedolizumab.

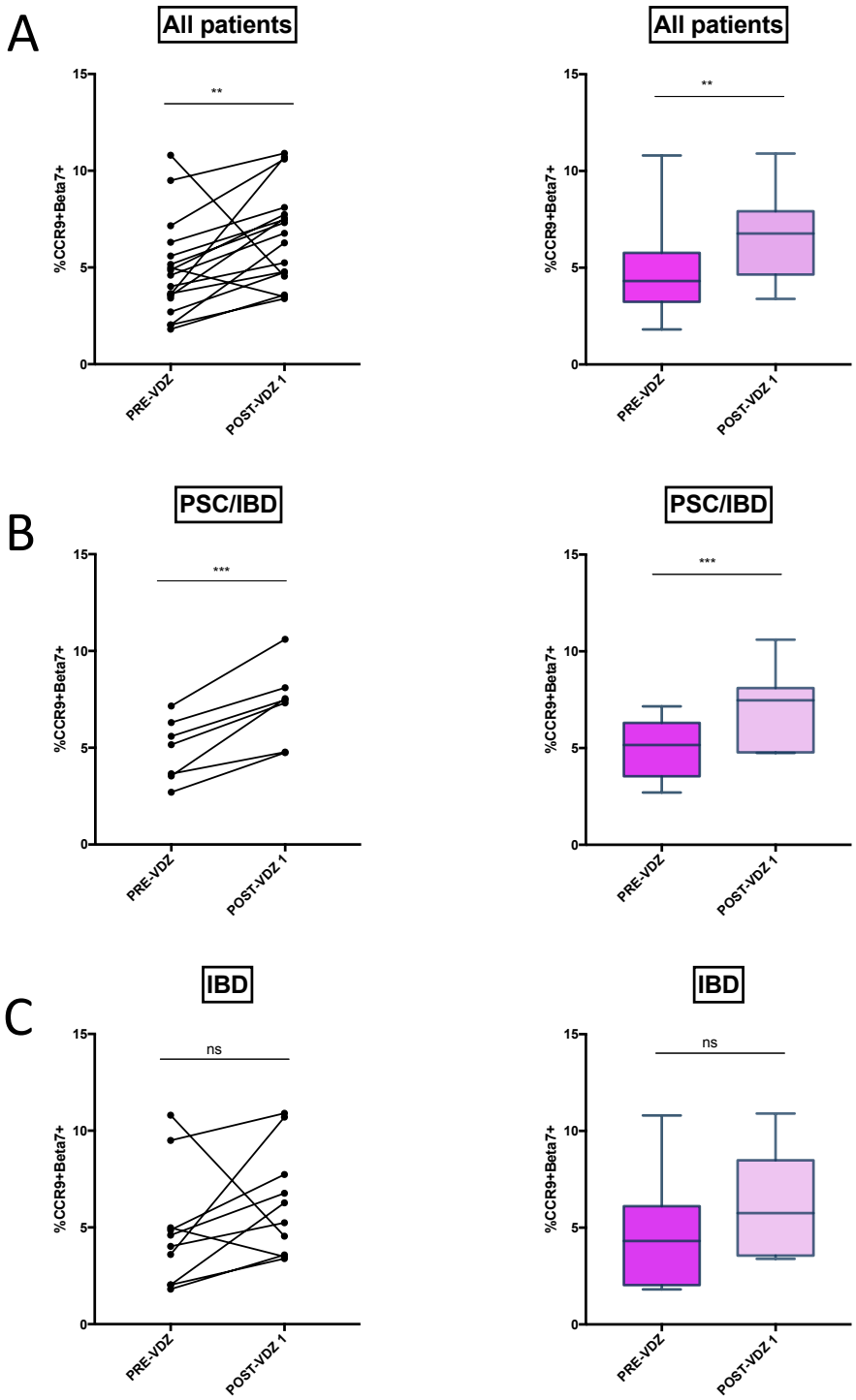


Figure 6-15 - Proportion of peripheral CD4+ memory T-cells which are CCR9/B7 dual positive with and without VDZ therapy, with comparisons between PSC/IBD and IBD

Cryopreserved PBMC taken pre- and post- VDZ were thawed and underwent staining and flow cytometric analysis. Cells were gated on CD4+ CD45RA- T-cell populations and the proportion which were CCR9/Beta7 dual positive was determined. Left graphs show individual patient trajectories and the right graphs show median±IQR, minimum to maximum. A, The proportion of CD4+ CD45RA- T-cells which are CCR9/β7 dual positive increases with VDZ when analysing all patients together (n=17). B, This remains true for patients with PSC/IBD (n=7). C, it is no longer statistically significant and to a smaller extent among patients with IBD only (n=10). Wilcoxon matched-pairs signed rank test and paired t-test carried out as appropriate. IBD, inflammatory bowel disease; PBMC, peripheral blood mononuclear cells; PSC, primary sclerosing cholangitis; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

6.4.1.10 Associations of receptor expression on memory T-cells in the peripheral blood with endoscopic remission

Using antibody panel 4 with the β7-PE monoclonal antibody, the finding of remitters having a lower proportion of CD4+ T-cells which were β7+ compared with non-remitters was confirmed, though with the mean values roughly double those found in the earlier experiment with antibody panel 1. The mean proportion of CD4+ T-cells which were β7+ was 29.0% (±SEM 2.6) in remitters vs. 46.2% (±16.7, p=0.016) in non-remitters. Whilst remitters had a numerically lower proportion of CD8+ T-cells which were β7 (40.8%±4.7) than non-remitters (56.2%±6.7, p=0.094), this finding did not reach statistical significance.

However, there was no difference in β7 expression among memory CD4+ T-cells between remitters and non-remitters with regards to β7 expression, though there was a difference seen among CD8 memory T-cells (see *Figure 6-16*). Remitters had a lower proportion of CD8+ CD45RA- T-cells which were β7+ (mean 44.7%±6.1) vs. non-remitters (20.4%±4.4, p=-0.008). There was no difference in the baseline CCR9 expression among memory T-cells between remitters and non-remitters, nor for CCR9/β7 co-expression (data not shown for the latter).

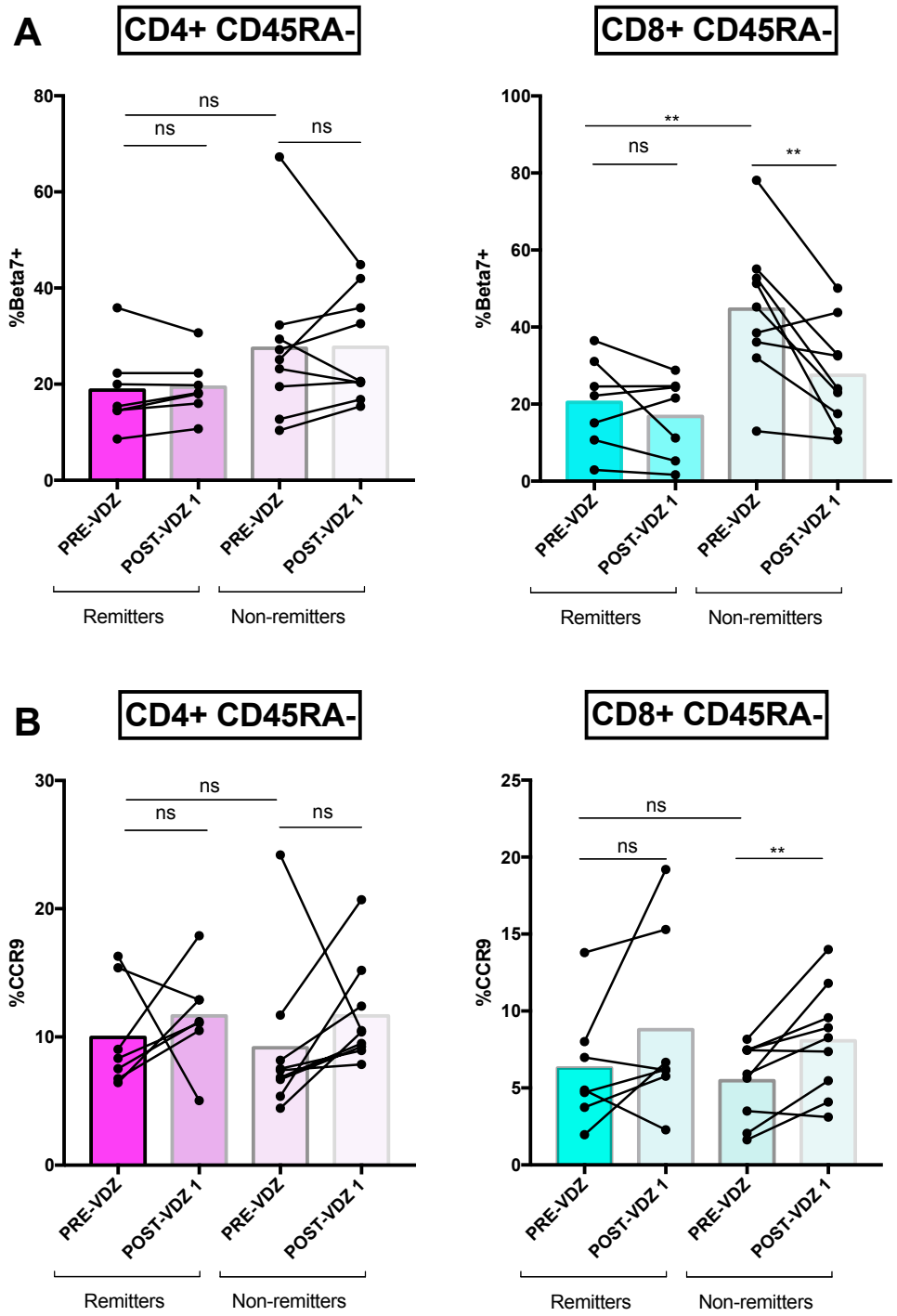


Figure 6-16 - Expression of β7 and CCR9 on peripheral memory T-cells in remitters and non-remitters on vedolizumab.

Flow cytometric analysis was performed on thawed PBMC taken from patients pre- and post-VDZ. The proportion of CD4+ CD45RA- T-cells (left) and CD8+ CD45RA- T-cells (right) which were β7+ (A) and CCR9+ (B) was determined. Changes pre- versus post-VDZ were analysed within the subgroups of patients who had undergone endoscopic remission (remitters, n=7) and who had not (non-remitters, n=9). The baseline concentrations of β7+ and CCR9+ cells were also compared between remitters and non-remitters. Bars represent mean values, and lines document trajectory of each patient. Paired t-test and Wilcoxon matched-pairs signed rank test were used as appropriate for paired analyses. For unpaired analyses, unpaired T-test and Mann Whitney test were used as appropriate. PBMC, peripheral blood mononuclear cells; VDZ, vedolizumab.
*p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

6.4.1.11 Summary of peripheral blood changes with vedolizumab

Below is a summary of the main results described above in the peripheral blood, which shall be discussed later in the chapter:

- CD4+/ CD8+ T-cells:
    - Proportion which are β7+ goes down with VDZ therapy.
    - This decrease occurs as early as day 14 after the first infusion, and persists to day 120+.
    - β7+ MFI reduces, particularly on the CD4+ β7+ population.
    - Patients with PSC/IBD have a greater drop in β7+ proportion compared with patients with IBD alone.
    - Remitters have a lower baseline β7+ proportion compared with non-remitters
- Memory CD4+ and CD8+ T-cells (i.e. CD45RA-):
    - There is no change in proportion of β7+ cells among CD4+ CD45RA- T-cells
    - Very small reduction in β7+ cells among CD8+ CD45RA- T-cells
    - There is a significant reduction in MFI of β7, with loss of intense expression of the β7 receptor
    - There is an increase in the proportion of CCR9+ cells
    - There is an increase in the proportion of CCR9/β7 dual positive cells among CD4+ CD45RA- cells
    - Only patients with PSC/IBD have a rise in CCR9 and CCR9/β7 dual positive cells (not patients with IBD alone)
    - There is no difference in remitters versus non-remitters with regards to β7 and CCR9 expression.

6.4.2 *Intestinal analysis: changes to β7 and associated receptor expression with vedolizumab*

Of the cohort described in 6.4.1.1, paired intestinal samples pre- and post-VDZ were available for 15 patients: 9 with PSC/IBD (all had UC) and 6 with IBD (5 UC, 1 CD). Samples were taken at endoscopy. Where possible, samples were taken from the ileum, inflamed colon and uninflamed colon.

LPMC were isolated from the intestinal biopsies and stained with antibody panel 1. As the majority of T-cells in the lamina propria are CD45RA-, the fact that this cell surface marker was not in the panel was not felt to be an undue limitation.

6.4.2.1 Effect of vedolizumab on ileal T-cell populations

Ileal samples were available for 10 patients pre-VDZ and 13 patients post-VDZ. Paired samples were available for 8 patients. Whilst there was a numerical reduction in the proportion of ileal CD4+ T-cells which were β7+ after VDZ, this reduction did not reach statistical significance (see *Figure 6-17*). This was the case whether analysing all the data (unpaired analysis) or paired data only. 6/8 had a reduction in the proportion of ileal CD4+ T-cells which were β7+. There was nothing to set apart the 2 patients whose β7+ proportion rose – both had PSC, and one went into remission whilst one did not. There was no difference in the proportion of ileal CD8+ T-cells which were β7+.

There was difference in CCR9 or CD161 expression on T-cell subsets with VDZ therapy.

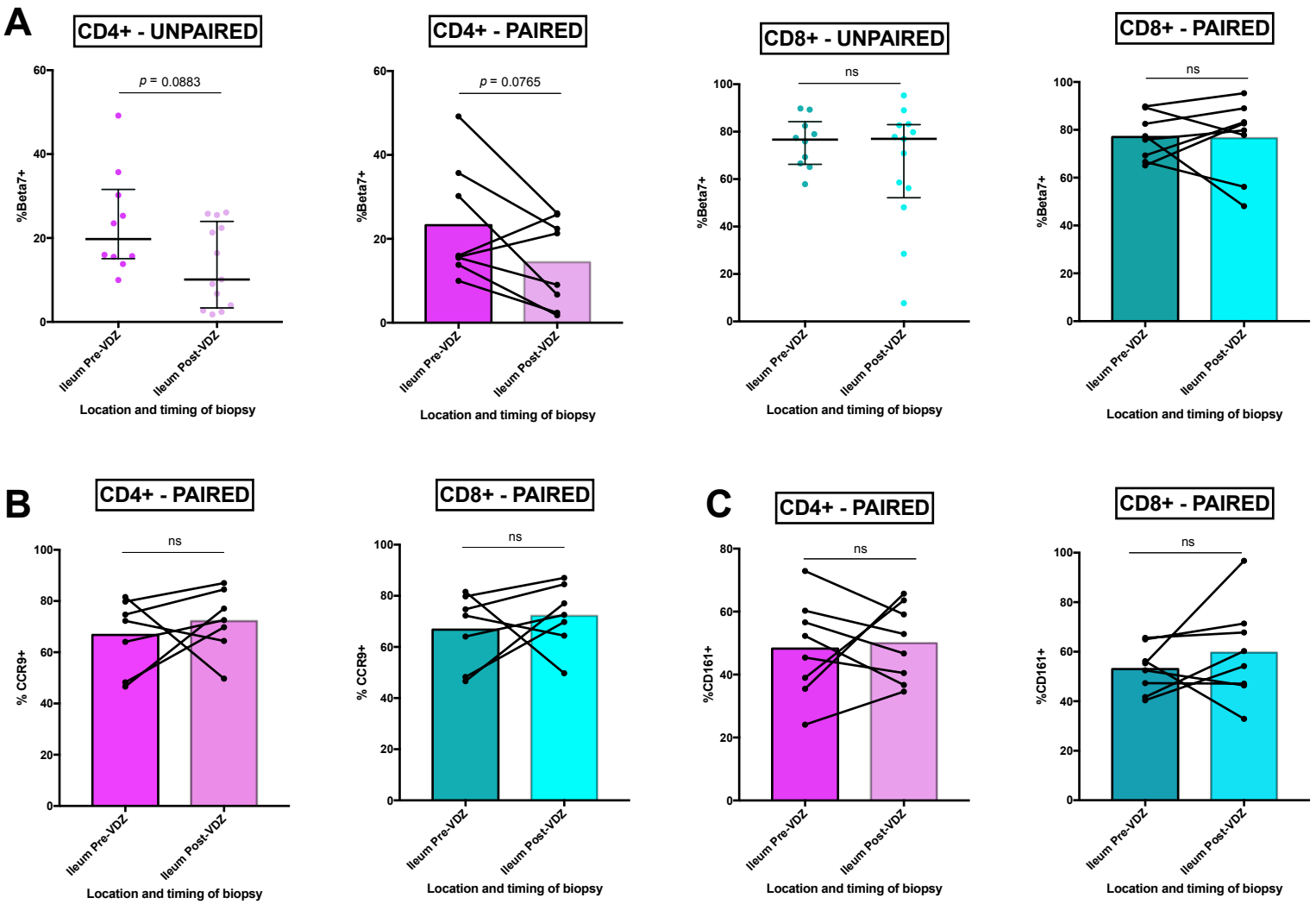


Figure 6-17 - β7, CCR9 and CD161 expression on ileal CD4+ and CD8+ T-cells pre- and post-VDZ therapy.

Legend – see next page.

Figure 6-17 – β7, CCR9 and CD161 expression on ileal CD4+ and CD8+ T-cells pre- and post-VDZ therapy.

[Previous page] LPMC were isolated from ileal biopsies from patients pre- and post-VDZ, and underwent flow cytometric analysis. The proportion of CD4+ T-cells (pink) and CD8+ T-cells (aqua) which were β7+ (A), CCR9+ (B) and CD161+ (C) was determined. In (A), both paired and unpaired analyses are shown, and though there was a small reduction in the percentage of CD4+T-cells which were β7+, this was not statistically significant. There were no differences pre- versus post-VDZ of CCR9 or CD161 expression either. The median ±IQR are depicted in the unpaired analyses, and the bars represent the mean in the paired analyses. Paired- t-test was used for the paired analyses, and Mann Whitney test for the CD4+ unpaired analysis and unpaired T-test for the CD8+ unpaired analysis. LPMC, lamina propria mononuclear cells; VDZ, vedolizumab; ns, not significant.

6.4.2.2 Effect of vedolizumab on colonic T-cell populations

Out of 15 patients, between 8 and 12 samples were available for each of uninflamed colon and inflamed colon, for time-points pre- and post-VDZ. In comparing colonic LPMC pre- and post-VDZ, where possible, the LPMC from inflamed colon samples were used, and for post-VDZ, LPMC from uninflamed colon. If these samples were not present for the pair, the other colonic sample was used. For example, if a patient had persisting colonic inflammation post-VDZ such that it was not possible to obtain uninflamed colonic samples, the inflamed colonic LPMC were used for the post-VDZ analysis for that patient. Substitution in this manner was only necessary for 4 patients. In this manner paired colonic samples were available for 14 patients.

The median proportion of colonic CD4+ T-cells which were β7+ decreased post-VDZ therapy from 14.8% (IQR 9.9-21.1) to 10.6% (IQR 6.2-16.1, p=0.035, see *Figure 6-18)*. 12/15 patients overall had a decrease in the percentage of CD4+ T-cells which were β7+. The three that had a rise in β7 proportion were all non-remitters.

There was no difference in the median proportion of colonic CD8+ T-cells which were β7+ post-VDZ.

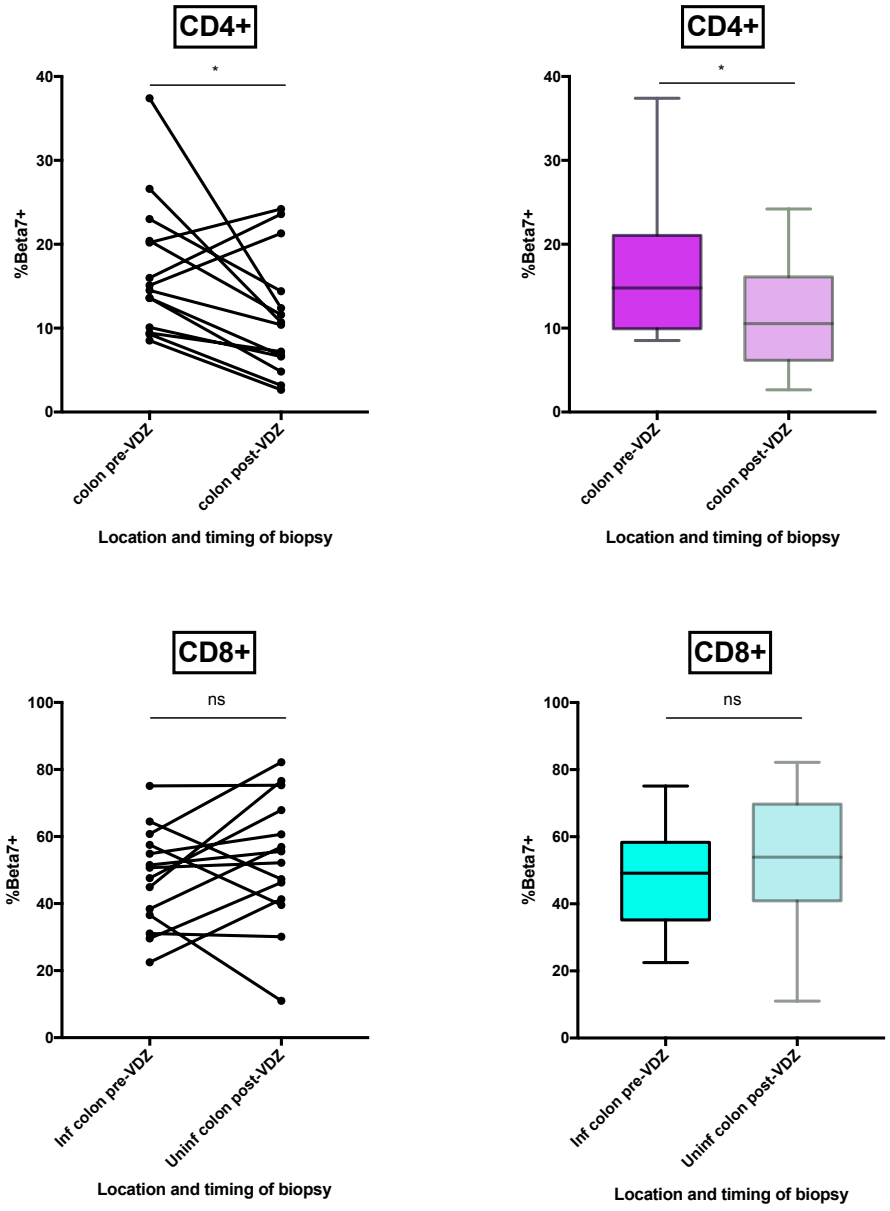


Figure 6-18 - Expression of β7 on colonic T-cells pre- and post-VDZ.

Colonic LPMC from samples pre- and post-VDZ were stained and underwent flow cytometric analysis. The proportion of CD4+ T-cells (upper graphs) and CD8+ T-cells (lower graphs) which were β7+ was determined. The left-hand graphs show each patient's trajectory pre- versus post-VDZ, whilst the right-hand graphs depict the median, IQR and minimum to maximum. The median proportion of β7+ cells reduced among colonic CD4+ T-cells, but not among colonic CD8+ T-cells. Wilcoxon matched-pairs signed rank test used for CD4+ analysis, paired t-test used for CD8+ analysis. LPMC, lamina propria mononuclear cells; VDZ, vedolizumab.
*=p<0.05, ns=not significant.

The mean proportion of colonic CD4+ and CD8+ T-cells which were CCR9+ increased numerically overall but not to statistical significance (see *Figure 6-19*). There was a rise in the proportion of CD4+ and CD8+ T-cells which were CD161+ post-VDZ. However, it was shown earlier that there is a higher proportion of CD161+ T-cells in uninflamed colon compared with inflamed colon. The fact that the analysis was primarily examining inflamed colon pre-VDZ and inflamed colon post-VDZ may have biased this finding. If one analyses paired uninflamed samples pre- and post-VDZ, or inflamed samples pre- and post-VDZ, there is no difference in CD161 expression.

6.4.2.3 Differences between PSC/IBD and IBD alone with regards to the effects of VDZ on colonic CD4+ and CD8+ T-cells

Although overall the mean proportion of colonic CD4+ T-cells which were β7+ reduced pre- versus post-VDZ, if one separates this into patients with PSC/IBD versus IBD alone, this finding was only significant for patients with PSC/IBD (see *Figure 6-20*). There was a drop from a mean 18.8% (±SEM 3.0) pre-VDZ to 11.0% (±2.3, p=0.022) in patients with PSC/IBD (n=8) compared with 14.3% (±2.8) to 12.0% (±3.5, p=0.544) in patients with IBD (n=6). All patients with PSC/IBD had a decrease, except for a single patient with a small rise, who had SD-PSC rather than classical PSC. There was no difference in β7 expression among colonic CD8+ T-cells for PSC/IBD vs. IBD.

Similarly, though there was no statistically significant difference in CCR9 expression on colonic T-cells pre- versus post-VDZ, for both CD4+ and CD8+ T-cells, there was a rise in the mean proportion which were CCR9+ for both CD4+ and CD8+ T-cells among PSC/IBD patients and a non-significant small drop for IBD patients.

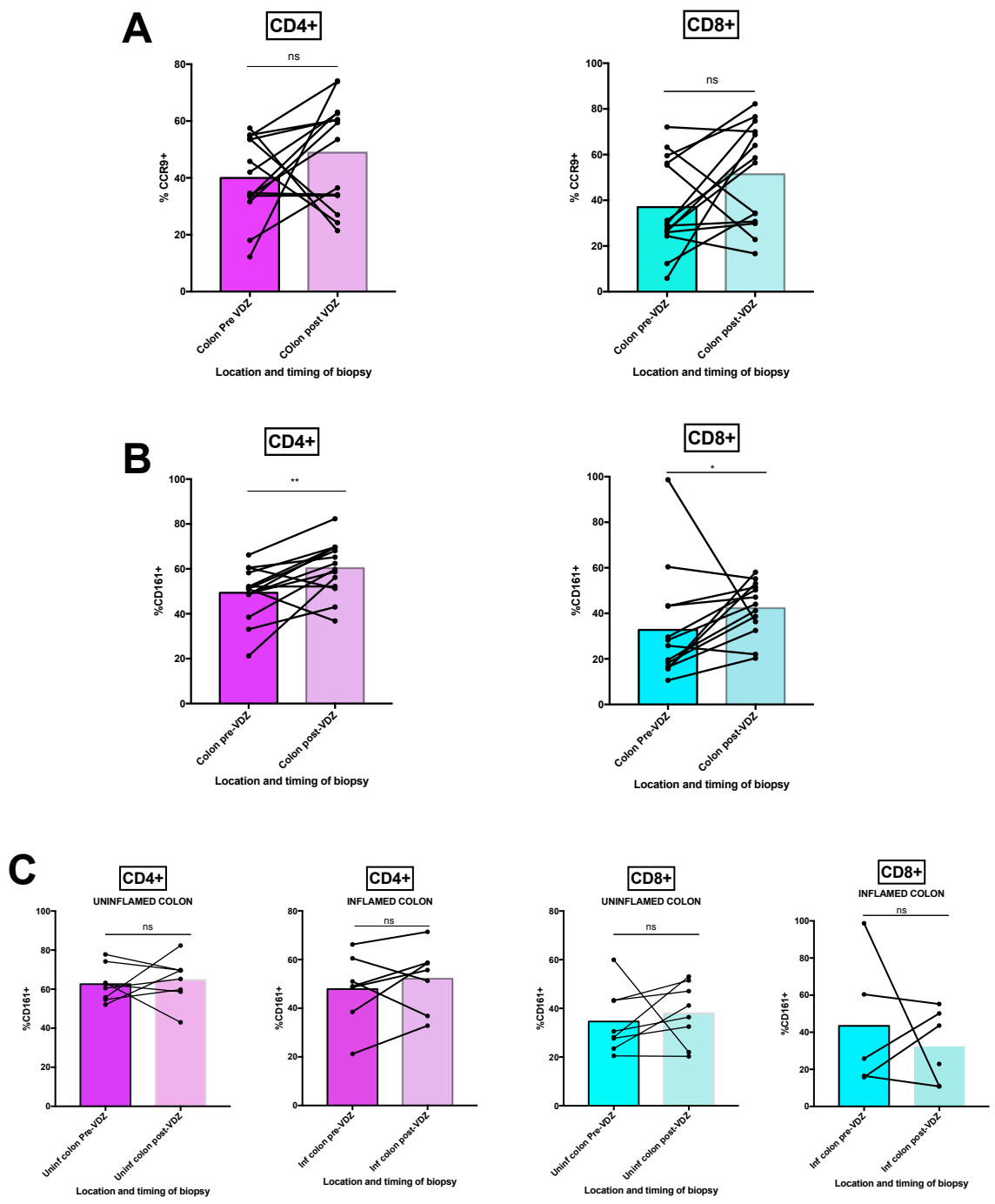


Figure 6-19 - Expression of CCR9 and CD161 on colonic T-cells pre- and post-VDZ.

LPMC were isolated from colonic samples pre- and post-VDZ. These were stained and underwent flow cytometric analysis. The proportion of T-cells which were (A) CCR9+ and (B) CD161+ were compared pre- and post- VDZ. In (A) and (B) for the pre-VDZ sample, data from inflamed colonic samples were used where possible (n=12), and substituted with data from uninflamed colonic samples if need be (n=2). Paired colonic samples post-VDZ were used for paired analysis, and where possible, data from uninflamed colonic samples were used (n=12) and substituted with data from inflamed colonic samples if need be (n=2). C, A subset analysis of paired inflamed samples pre- and post- VDZ and paired uninflamed samples pre- and post-VDZ shows no difference in CD161 expression. Pink graphs show CD4 populations, and aqua graphs show CD8 populations. Each patient trajectory is shown by spaghetti plots, and mean is depicted by bars. LPMC, lamina propria mononuclear cells; inf, inflamed; uninf, uninflamed; VDZ, vedolizumab. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

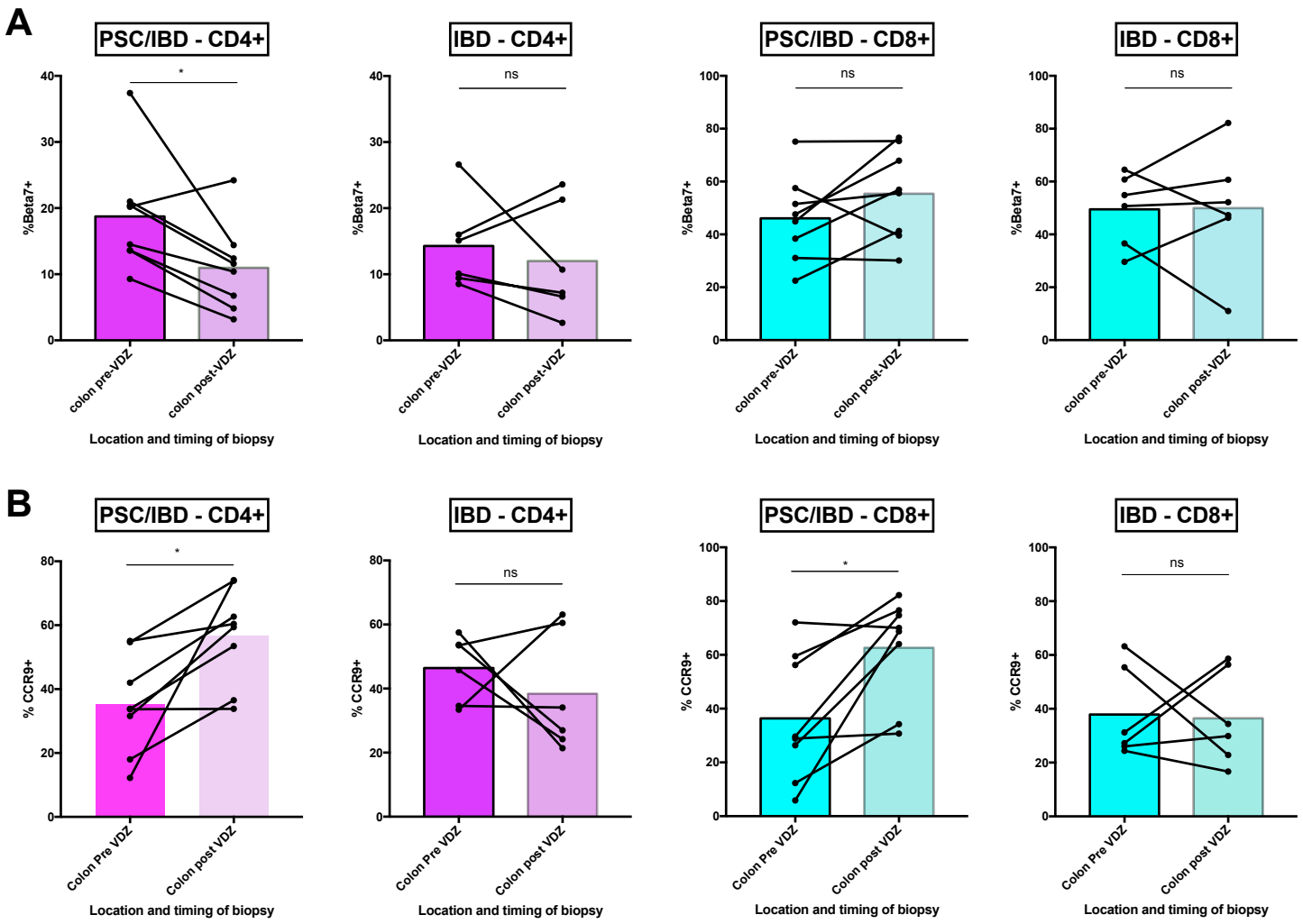


Figure 6-20 - PSC/IBD vs. IBD: β7 and CCR9 expression on T-cells pre- and post-vedolizumab.

Legend (see next page)

Figure 6-20 - PSC/IBD vs. IBD: β7 and CCR9 expression on T-cells pre- and post-vedolizumab

[Previous page] The proportion of colonic CD4+ (pink graphs) and CD8+ T-cells (aqua graphs) which were β7+ (A) and CCR9+ (B) were compared pre- versus post-VDZ in patients with PSC/IBD (n=8) and IBD alone (n=6). Patients with PSC/IBD had a reduction in the mean proportion of CD4+ T-cells which were β7+ whilst those with IBD alone did not. Similarly, patients with PSC/IBD had a rise in the mean proportion of CD4+ and CD8+ T-cells which were CCR9+ whilst those with IBD alone did not. Individual trajectories pre- to post-VDZ are shown by the spaghetti plots, and the bars represent the mean value. VDZ, vedolizumab. *p=0.01-0.05; **p=0.001-0.01; ***p=0.0001-0.001; ****p<0.0001; ns=not significant, i.e. p>0.05.

6.4.2.4 Interpretation of results of colonic and peripheral blood β7 and CCR9 expression with vedolizumab

Taking the peripheral blood finding and colonic findings together, after administration of VDZ, patients with PSC/IBD had a rise in CCR9+ T-cells in both the peripheral blood and the colon of CCR9+ T-cells together with a reduction in colonic β7+ CD4+ T-cells. Whilst some patients with IBD alone (i.e. no PSC) did individually have these changes, overall there was no statistically significant change, and the changes were smaller numerically.

The cells being targeted (CCR9+ and CCR9/β7 dual positive) mark out the gut-homing cells which have the highest β7 expression – usually the CD4+ memory T-cells. There was no difference in CD4+ CD45RA- β7+ T-cells with VDZ, but this may be due to internalisation of the α4β7 receptor, which prevents their being detected by cell surface staining with the β7 monoclonal antibody. However, CCR9 may be a surrogate marker to detect them, and it is likely that cells which have an initial high expression of β7 (such as CD4+ CD45RA- T-cells) have some β7 still expressed at the surface after VDZ due to a higher number of receptors overall, and therefore they still remain detectable by flow cytometry with cell-surface staining. Hence, the proportion of β7+ cells does not change among the CD4+ CD45RA- population, but it reduces among the overall CD4+ population (where the

majority of cells have low to intermediate β7+ expression, and internalisation of the α4β7 receptor renders them no longer detectable by β7 surface staining).

Thus, from these data, one might conclude that VDZ has its greatest effect on gut-homing T-cells, namely CD4+ CD45RA- T-cells, which have the highest expression of α4β7 and co-express CCR9, and cause these cells to remain within the peripheral blood with a corresponding reduction in the colon. Furthermore, this effect is accentuated in patients with PSC/IBD compared with IBD alone for reasons that are not altogether clear.

6.4.2.5 Associations of receptor expression on colonic T-cells with endoscopic response and remission

Of 14 patients with paired colonic biopsies, endoscopic response and remission data were available for 12. One patient had Crohn's disease and one patient had a baseline UCEIS of 1/8, hence UC response/ remission could not be calculated for these two patients. 7/12 patients had an endoscopic response and 5/12 patients achieved endoscopic remission.

With regards to the proportion of colonic CD4+ T-cells which were β7+, this reduced in 6/7 responders and 3/5 non-responders (see *Figure 6-21*). The one responder whose proportion of β7+ cells increased post-VDZ had an endoscopic response but did not achieve an endoscopic remission (UCEIS 2 at post-VDZ, from 5/8). If one compared the pre- versus post-VDZ median proportion of CD4+ β7+ cells, this decreased to almost half the baseline values (13.6% [IQR10.1-20.4]) to 7.2% [IQR6.6-11.6], p=0.1094) but this change did not reach statistical significance. Non-responders had negligible change. There was no difference in baseline β7 expression among colonic CD4+ T-cells between responders and non-responders.

When considering endoscopic *remitters* versus *non-remitters*, all 5 remitters had a reduction in the proportion of colonic CD4+ T-cells which were β7+ compared with only 4/7 non-remitters. Remitters dropped from a baseline mean proportion of 13.4% (±SEM 1.9) to 7.4% (±1.1, p=0.011). Non-remitters had a negligible non-significant drop: mean 17.8% (±2.2) to 15.4% (±3.0, p=0.490). The proportionate change (described as the difference in β7+ cells as a percentage of the baseline proportion) was a mean -43.2% (±7.0) in remitters versus -11.8% (±17.9) for non-remittters, but this difference did not reach statistical significance (p=0.188).

Numerically, remitters had a slightly lower mean proportion of colonic CD4+ T-cells which were β7+ at baseline (13.4% ±1.9) compared with non-remitters (17.8% ±2.2) but this did not quite reach statistical significance (p=0.186, see *Figure* 6-22). Furthermore, the mean proportion of β7+ cells post-VDZ was roughly half in remitters (7.4% ±1.1) compared with non-remitters (15.4% ±3.0), but again, this did not quite reach statistical significance (p=0.057).

There was no correlation between the proportion of colonic CD4+ T-cells which were β7+ on from inflamed colon pre-VDZ with the UCEIS at baseline (i.e. at the same time as sampling, see *Figure 6-23)*. There was also no correlation between this proportion of β7+ cells at baseline and post-VDZ UCEIS.

Given that there were only 12 patients being analysed, it is possible that some of these non-significant findings are actually true findings, but the sample size was not large enough to adequately detect a difference. It must also be noted that the numerical values

of the proportions of β7+ cells detailed here are likely to be underrepresented due to the utilisation of a weaker fluorochrome on β7 (PerCP-Cy5.5 – antibody Panel 1), though the proportionate comparisons are likely to be correct.

There were no predictive factors of endoscopic response or remission when analysing colonic CD8+ T-cells, nor analysing CCR9 expression on colonic T-cells.

6.4.2.6 Interpretation of colonic data

These data, though not conclusive, suggest that VDZ does indeed reduce the amount of β7+ T-cells in the colon, and, furthermore, a greater reduction is associated with endoscopic remission. This is the first study to date to lend proof of concept evidence to the mechanism in which VDZ is purported to work – that is, by reducing trafficking of gut-homing T-cells to the colon. It challenges the notion by Zeissig *et al.* that VDZ does not reduce T-cell trafficking to the colon, as mentioned in *Section 1.6.7*.[169] This dichotomy in findings may be due to the fact our study had almost double the patients compared with the Zeissig study, and their study may have been underpowered. It perhaps may also have been affected by not enough patients entering remission. They analysed a different outcome also – they analysed the number of T-cells as a percentage of total cells within the lamina propria, both by IHC and flow cytometry. A reduction in T-effector cells can lead to a reduction in stimulatory cytokines, which would bring about a reduction in recruitment of innate and adaptive effector cells. Hence, all cell populations would reduce and looking at the overall proportion of T-cells may not be the ideal way to look at the effect of VDZ. Rather, we have shown that VDZ reduced the proportion of CD4+ T-cells which are β7+, as a surrogate for α4β7+, which would therefore lead to a reduction in the recruitment of T-cells to the colon, and subsequent resolution of inflammation.

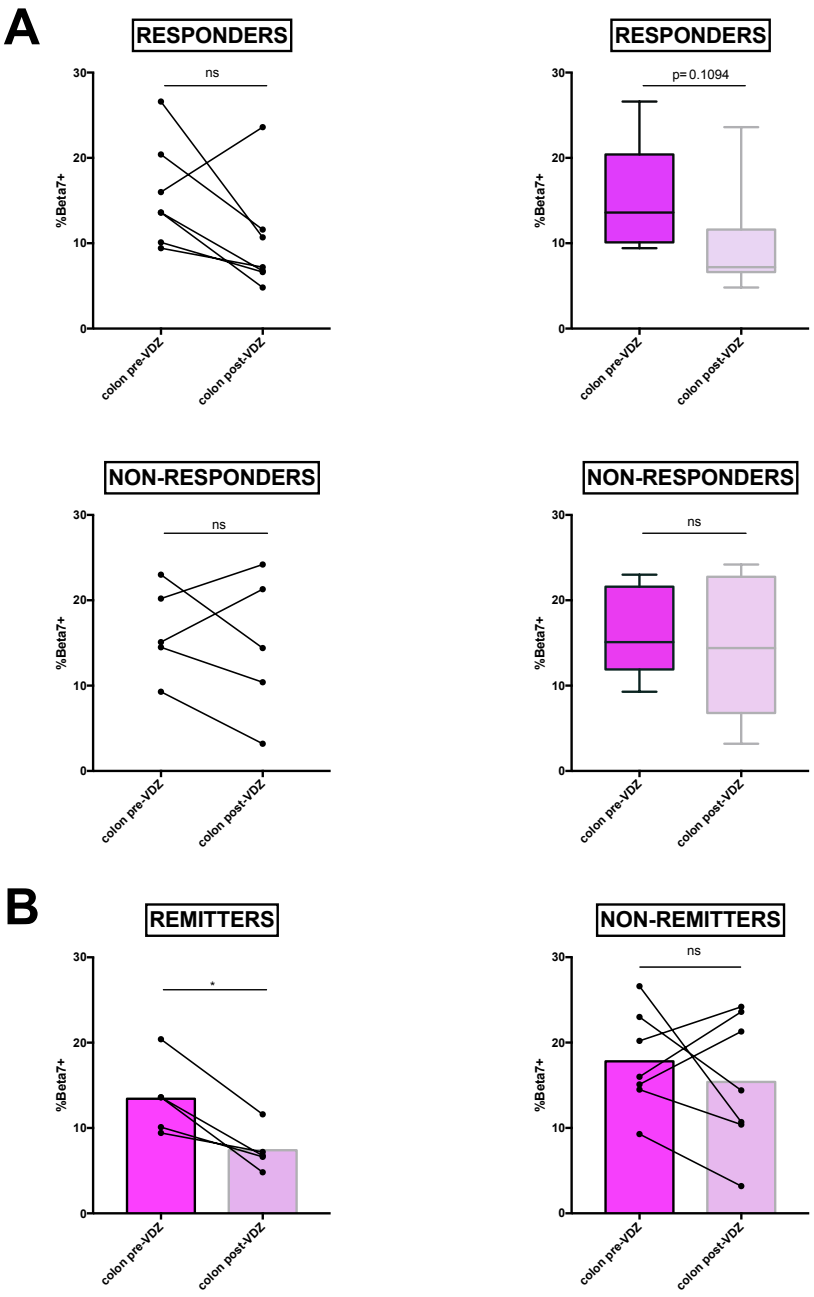


Figure 6-21 - Changes in b7 expression among colonic CD4+ T-cells according to endoscopic response and remission

The proportion of colonic CD4+ T-cells which were (7+ was determined via flow cytometry pre- and post-VDZ. A, responders are shown in the upper panel, and non-responders are shown in the lower panel. The left-hand graphs depict individual patient trajectories pre- to post-VDZ and the right-hand graphs show the median±IQR and range. B, Mean values shown and individual trajectories for remitters (left) and non-remitters (right). C, The percentage change in (7+ cells taken as the absolute difference as a percentage of the baseline. Paired t-test or Wilcoxon matched-pairs signed rank test applied as appropriate. VDZ, vedolizumab. *=p<0.05, ns=not significant.

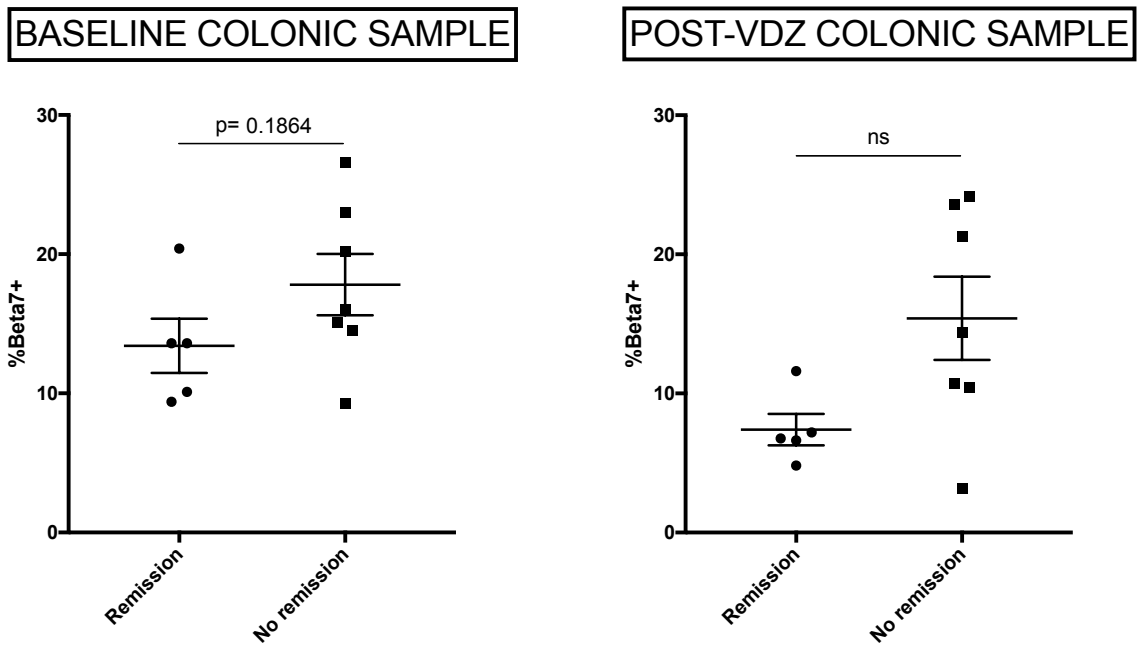
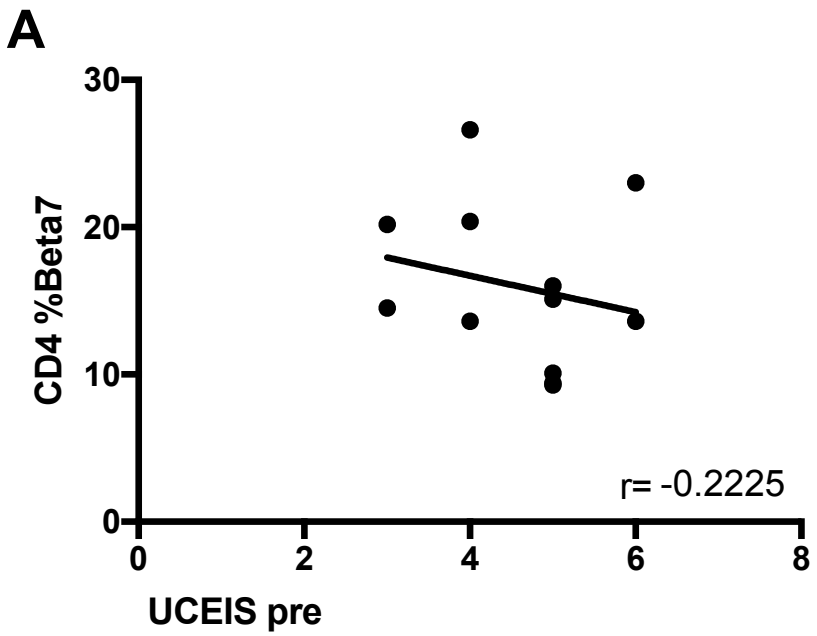


Figure 6-22 - Remitters vs. non-remitters: differences between baseline and post-VDZ colonic β7 expression

The proportion of colonic CD4+ T-cells which were β7+ was determined via flow cytometry at baseline (left-hand graph) and post-VDZ (right-hand graph). The values were compared between remitters and non-remitters (remission = UCEIS≤1 at post-VDZ colonoscopy). Mean ±SEM is shown. Paired t-test applied. ns, not significant; VDZ, vedolizumab.



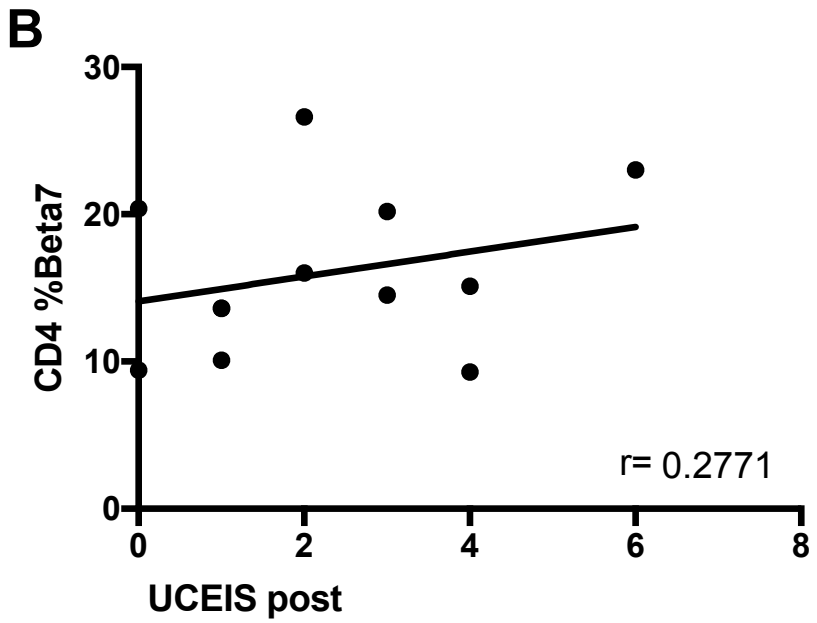


Figure 6-23 - Correlation between proportion of colonic CD4+ T-cells at baseline colonoscopy and UCEIS.

The proportion of colonic CD4+ T-cells which were β7+ at baseline endoscopy were determined via flow cytometry. Patients endoscopic score, UCEIS, was determined at the same endoscopy at baseline as well as at an endoscopy post-VDZ. Higher scores indicate a greater degree of inflammation, and lower scores indicate more quiescent disease. A, There was no correlation between the proportion of β7+ CD4+ T-cells (taken as a proportion of total CD4+ T-cells) and the UCEIS at baseline. B, There was also no correlation between the proportion of colonic CD4+ T-cells which were β7+ at baseline and the subsequent UCEIS at follow-up endoscopy. Linear regression was performed with a line of best fit inserted. Pearson r correlation was determined. UCEIS, ulcerative colitis endoscopic index of severity; VDZ, vedolizumab.

6.4.3 *Liver analysis β7 and associated receptor expression in the liver after therapy with vedolizumab*

Liver infiltrating lymphocytes (LILs) were available from 5 patients who were on VDZ therapy for their IBD. 4 patients had a form of PSC (3 PSC, 1 SD-PSC) and 1 patient had NASH. Three patients had UC, and 2 had IBD-u. All patients were male. Baseline demographics are shown in *Table 6.5*.

Three patients had had a rise in their liver enzymes beyond what would be expected for the natural history of their disease and therefore underwent a liver biopsy for clinical purposes. One patient had a liver biopsy at the time of his cholecystectomy to stage his liver disease, and one patient had an FNA through "The PSC FNA Liver Study" but had normal liver biochemistry at the time. For three patients, LILs were derived from an FNA and for two patients, they were derived from a biopsy.

All patients who had undergone an FNA or liver biopsy and had adequate flow cytometric analysis of the LILs were analysed with regards to whether VDZ affected β7 or CCR9 expression on memory T-cells. Given no true difference between patients with PSC and liver controls was found with regards to β7 and CCR9 expression, the samples were combined regardless of underlying disease, and separated into two cohorts: those on VDZ (n=5) and those not on VDZ (n=31).

Table 6.5 - Baseline demographics of patients on vedolizumab undergoing liver biopsy or fine needle aspiration.

ID	Age (y)	Sample used*	Liver disease	IBD	VDZ duration (days)	ALT at bx (IU/L)	Peak ALT on VDZ (IU/L)	ALP at bx (IU/L)	Peak ALP on VDZ (IU/L)	Unexpected rise in LFTs
FNA#11	48.8	FNA	NASH[#]	IBD-u	715	44	217	293	320	Y
FNA#23	25.7	FNA	SD-PSC	IBD-u	457	73	161	322	668	Y
FNA#37	31.8	FNA	PSC	UC	881	22	33	59	67	N
IBD#2174	25.2	Bx	PSC	UC	294	172	294	1188	1373	Y
IBD#1370	23.2	Bx	PSC	UC	702	157	255	447	490	N

*This indicates the type of sample used to isolate the liver infiltrating lymphocytes for analysis, though the patient may have also had a liver biopsy at the same time for diagnostic purposes. [#]This patient only had NASH on biopsy with a normal MRCP, but a repeat MRCP has shown subtle intrahepatic duct irregularities, raising the possibility of PSC which may have been missed on liver biopsy due to the patchy nature of the disease.
ALP, alkaline phosphatase; ALT, alanine transaminase; Bx, biopsy; FNA, fine needle aspiration; IBD, inflammatory bowel disease; IBD-u, inflammatory bowel disease-unspecified; IU/L, international units per litre; LFTs, liver function tests; N, no; NASH, non-alcoholic steatohepatitis; M, male; MRCP, magnetic resonance cholangiopancreatography; UC, ulcerative colitis; VDZ, vedolizumab; Y, yes.

There was no difference in β7 expression on CD4+ or CD8+ memory T-cells within the liver between patients on VDZ and patients not on VDZ. However, when analysing the proportion of T-cells which were CCR9+, there was a significant difference between those on VDZ and those not on VDZ.

The mean proportion of liver-derived CD4+ memory T-cells which were CCR9+ was 13.9% (±SEM2.1) for patients on VDZ compared to 4.3% (±0.4, $p<0.0001$) for patients not on VDZ (see *Figure 6-24*). All patients who were on VDZ had a CCR9+ proportion higher than any patient not on VDZ, except for one patient (FNA#37). His proportion of CCR9+ CD4+ memory T-cells (8.2%) was still higher than the mean of patients not on VDZ, but overlapped with some of the patients not on VDZ. Notably, FNA#37 had normal liver biochemistry at the time of sampling, compared with the other four patients on VDZ who had raised liver biochemistry.

This difference in CCR9 expression was also found among CD8+ memory T-cells. The mean proportion of liver-derived CD8+ memory T-cells which were CCR9+ was 7.3% (±1.1) for patients on VDZ versus 2.9% (±0.4, $p=0.004$) for those not on VDZ. There was some overlap of values between those not on VDZ (higher values) and those on VDZ (lower values). Once again, the lowest proportion of CCR9+ cells among the VDZ cohort was FNA#37.

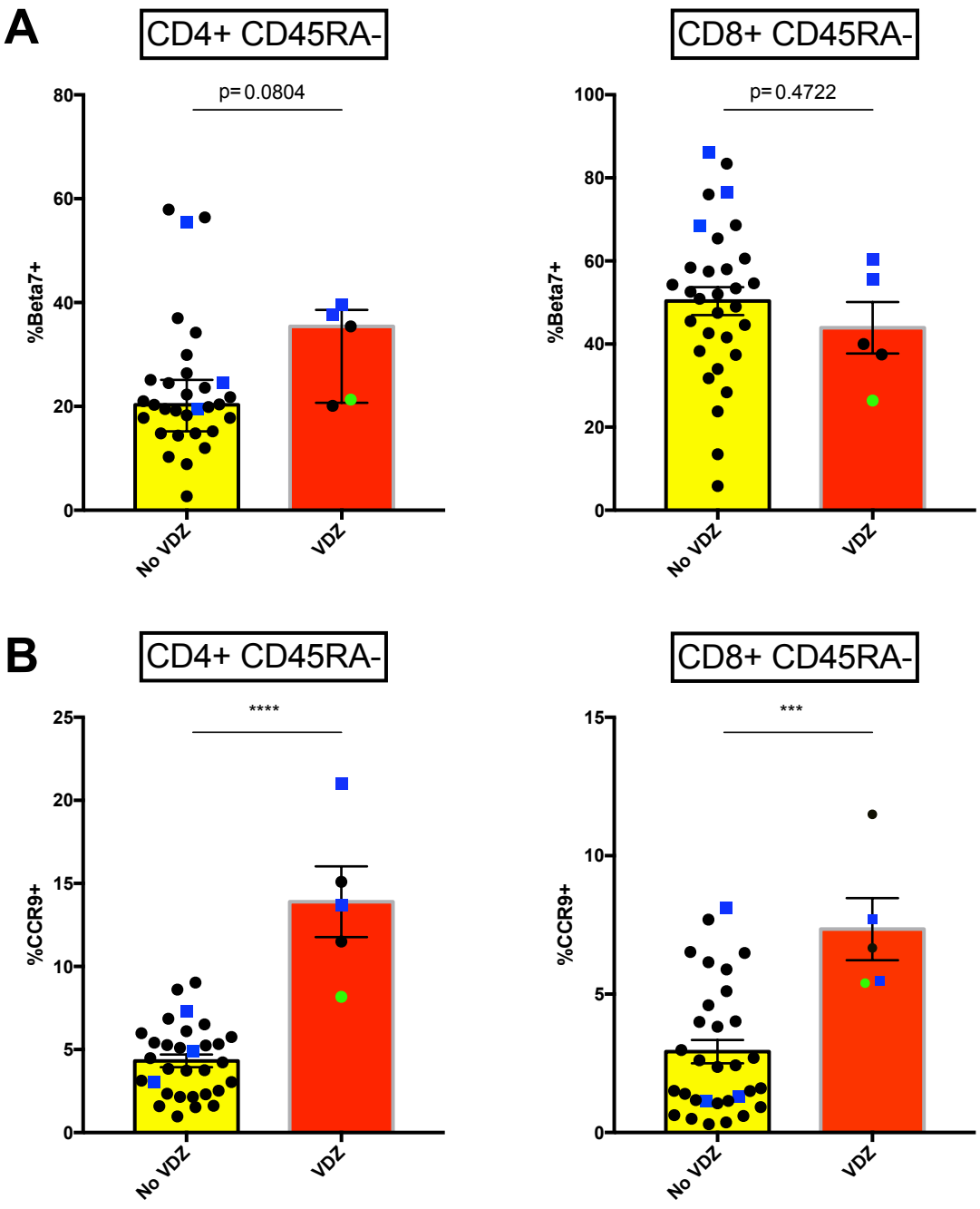


Figure 6-24 - The proportion of liver memory CD4+ and CD8+ T-cells which are β7+ and CCR9+ according to VDZ use.

Liver infiltrating lymphocytes were isolated from patients who underwent either FNA (circles) or liver biopsy (blue squares). Patients either had PSC/UC or another liver disease. Patients were separated into those on VDZ (n=5) and those not on VDZ (n=31). The proportion of CD4+ memory cells (left-hand graphs) and CD8+ memory T-cells (right-hand graphs) which were positive for β7 (A) and CCR9 (B) were compared between the two cohorts. There was no difference between cohorts with regards to β7 expression. However, patients on VDZ had a significantly higher proportion of CD4+ and CD8+ memory T-cells which were CCR9+. The green dot represents FNA#37 who had PSC/UC and was on VDZ but had normal liver biochemistry at the time of sampling. All graphs show the mean (bars) and SEM, and unpaired t-test has been applied, except for the left-hand graph in (A) which shows the median (bars) and IQR, and Mann-Whitney test was applied. FNA, fine needle aspirate; PSC, primary sclerosing cholangitis; UC, ulcerative colitis; VDZ, vedolizumab.

6.5 Discussion and summary of findings

These data show a clear relative abundance of CCR9+ memory T-cells, particularly CD4+ memory T-cells, in the livers of patients on VDZ compared with those not on VDZ. Together with the raised CCR9+ and CCR9/β7 dual positive CD4+ CD45RA- T-cells in the peripheral blood post-VDZ, the most likely explanation is that VDZ causes truly does prevent gut-homing memory CD4+ T-cells leaving the blood (i.e. blocking entry to the gut), leading to increased availability for infiltration of these cells into the liver.

In the liver, lymphocytes are recruited to the parenchyma from sinusoids across LSECs.[221] In the sinusoids, there is mixing of oxygen rich hepatic arterial blood and nutrient rich portal venous blood. There is a low flow environment in the hepatic sinusoids, which, together with the discontinuous fenestrated endothelium, allows passage of nutrients, metabolites and oxygen across LSECs into the hepatic parenchyma. However, this also facilitates immune surveillance of the liver, with Kuppfer cells crawling across LSECs to detect any danger-associated molecular patterns (DAMPs) which may have been released due to injury from toxins or infection. This low shear environment, also means that lymphocyte recruitment to the liver is usually selectin-independent, and that no rolling/tethering step is usually required, unlike in other tissues, such as the gut.

Just as in the gut, lymphocyte recruitment is dependent on chemokine-activated integrin-mediated adhesion. As mentioned previously, CCL25 has been shown to be aberrantly expressed on sinusoidal endothelium in the livers of patients with PSC and is thought to be involved in the potential hepatic recruitment of gut-homing T-cells in PSC via binding of CCR9 to CCL25.[137] Increased passage of peripheral blood CCR9+ T-cells through the

hepatic sinusoids (due to VDZ therapy) may lead to increased binding to CCL25 in the PSC liver. Subsequent adhesion is usually then triggered through $\alpha4\beta7$-MAdCAM-1 interactions. However in this case, the $\alpha4\beta7$ receptor is likely occupied by VDZ, preventing binding with MAdCAM-1. It has been shown that $\alpha4\beta7$ also binds to the adhesion molecule, VCAM-1 and that vedolizumab does not prevent this interaction.[112, 116] Furthermore, VCAM-1 is present on human LSECs, and is expressed at higher levels on inflamed LSECs (such as, potentially, in PSC).[222, 223] Lastly, it has also been shown on flow-based adhesion assays of LSECs, that blockade of a single component of the adhesion cascade may reduce but does not completely prevent migration of T-cells across LSECs.[224] Taken together, it is possible that the CCR9+ T-cell may gain access to the liver parenchyma through an alternative adhesion pathway to $\alpha4\beta7$-MAdCAM-1 interaction. This may be via $\alpha4\beta7$-VCAM-1 interaction.

Another possible explanation of the raised CCR9+ T-cells within the liver on VDZ therapy, is that the sampling technique – homogenisation of a liver biopsy or a fine needle aspirate of the liver – may be rich in sinusoidal T-cells as well as parenchymal T-cells. The increased number of CCR9+ T-cells derived from the liver may be due to an increased sinusoidal load from the peripheral blood. However, even if it is due to sinusoidal rather than parenchymal T-cells, this may still represent an important change in the inflammatory milieu within the liver. There are many interactions between sinusoidal immune cells and the LSECs which can have physiological and pathological effects.[221]

It is interesting that patients who do not have underlying liver disease/ PSC who are exposed to vedolizumab do not have liver biochemistry rises to the extent that some

patients with PSC do. Indeed, in the GEMINI studies there was no signal that there was a significant proportion of patients who had a rise in liver biochemistry. And yet, in our international study of patient with PSC/IBD on VDZ, 41.2% had a rise in their ALP by 20% or more. This clear discordance between the changes in liver biochemistry which can occur in patients with PSC/IBD and patients with IBD alone may reflect the ability of the inflamed liver in PSC to recruit these redirected gut-homing T-cells within the peripheral blood where the healthy liver cannot. This could be due to the increased expression of CCL25 and VCAM-1 in the inflamed liver as described above.

All patients on VDZ had higher proportions of hepatic CD4+ memory T-cells which were CCR9+ than patients not on VDZ, with the exception of one patient. FNA#37 had only 8.2% of cells CCR9+ compared with the highest proportion among non-VDZ patients being 9.03% - a slight overlap. This particular patient – FNA#37 – had normal liver biochemistry at the time of liver sampling, and had not had a rise in his liver biochemistry after therapy with VDZ. This is despite his having had a rise in his proportion of CD4+ memory cells which were CCR9+ within the peripheral blood (from 8.3% to 11.1%). He may have had less active inflammation within his liver as reflected by his normal liver biochemistry, and therefore may not have had CCL25 and VCAM-1 upregulated to such a degree within his liver. Hence, whilst his proportion of CD4+ and CD8+ memory T-cells which were CCR9+ was above the mean compared with those not on VDZ, it was not as high as other patients on VDZ.

This may explain why I found in my Oxford VDZ study that patients with a normal ALP at baseline did not tend to have an ALP rise on VDZ, compared to those whose ALP was

above the ULN at baseline were more likely to have an erratic course and/or a rise in ALP with VDZ. Conversely, it could also simply reflect the natural course of the disease. Patients with a normal ALP have a more quiescent disease course with a better clinical prognosis, compared with those with an ALP persistently raised above 1.5xULN.

One way to learn more about this, and the differential handling of gut-homing T-cells in patients on VDZ, would be to analyse LILs in more patients with PSC/IBD, but to include patients who have normal liver biochemistry at baseline as well as those with raised biochemistry. My sample described here is only 5 patients (and one patient had NAFLD, though potentially may actually have underlying PSC), and is biased towards patients with raised liver biochemistry on VDZ.

The fact that CCR9+ CD4+ T-cells increase in the colon with VDZ, and more so in patients with PSC/IBD compared with IBD alone, is slightly counterintuitive given the above argument. If CCR9+ CD4+ T-cells are prevented from entering the gut with VDZ and remain within the blood, then there should be a *decrease* in the colon with VDZ. One explanation could be that CCR9+ $\alpha4\beta7$- CD4+ T-cells are gaining entry to the colon through some mechanism independent of $\alpha4\beta7$-MAdCAM-1 interactions. There are some CCR9+/$\alpha4\beta7$- CD4+ T-cells present within the peripheral blood, albeit not as many as dual positive. Or, CCR9 may be induced on the T-cells which are able to enter the colon.

In summary, in this chapter, I have described that VDZ causes significant redistribution of gut-homing T-cells in patients with PSC/IBD and IBD. The proportion of overall CD4+ and CD8+ T-cells which are $\beta7$+ goes down, regardless of clinical response, which may be due

to internalisation of the $\alpha 4\beta 7$ receptor on binding with VDZ. However, in particular, VDZ targets the CD4+ memory T-cells, significantly reducing the expression of $\beta 7$ (presumably by internalisation) and causing a rise of CCR9+ and CCR9/$\beta 7$ dual positive cells within the peripheral blood. This is particularly apparent in patients with PSC/IBD compared with IBD alone, though numbers were small in both groups.

This redistribution of gut-homing T-cells by VDZ leads to two important outcomes within the tissue. Firstly, the proportion of $\beta 7$+ T-cells within the colon reduces significantly, again in PSC/IBD more so than in IBD alone. This brings about a clinical response and sometimes remission in a proportion of patients. Patients who had the lowest expression of $\beta 7$ on colonic CD4+ T-cells post-VDZ tended to have a higher chance of endoscopic remission, though this finding did not quite reach statistical significance.

Secondly, in patients on VDZ, there is a higher proportion of CCR9+ memory T-cells within the liver, particularly CCR9+ CD4+ memory T-cells, compared with patients not on VDZ. There is a trend towards an increase in $\beta 7$+ CD4+ T-cells within the liver with VDZ. These findings suggest that VDZ causes a redirection of gut-homing T-cells from the intestine to the liver in selected patients with underlying liver disease, mainly PSC/IBD, and particularly in those with elevated liver enzymes. Further analysis of liver-derived T-cells pre- and post-VDZ in a larger cohort of patients with PSC/IBD, and potentially patients without underlying liver disease, may help to further elucidate the effect of VDZ on the passage of gut-homing T-cells through the body.

7 Conclusion

This thesis has analysed a large cohort of patients with PSC (and often concomitant IBD) with respect to the phenotype and distribution of their gut-homing lymphocytes and the effect of a therapy targeting these T-cells, and compared these data with those from control cohorts. Previous studies have mainly analysed patients who are at the end stage of disease, particularly with respect to LILs, and these data add significant new findings to the PSC literature.

The principal findings in this thesis are:

1. Patients with PSC have a reduced proportion of CCR9+ and CCR9/β7+ T-cells within the peripheral blood compared to controls (UC).
2. Patients with PSC have reduced CCR9+ T-cells within their ileum compared to controls (UC and HC).
3. There is a subset of patients, both with PSC and other chronic liver diseases, who have a significant proportion of CCR9+ CD4+ T-cells within their liver, and they reside within the portal tracts.
4. These patients trend towards having higher markers of fibrosis and inflammation.
5. There is no difference in the proportion of CCR9+ T-cells in the liver in PSC compared to LCs.
6. Patients with PSC have increased MAITs within their liver as compared to LC.
7. Liver FNA is a safe and well-tolerated technique which adequately samples a distinct lymphocyte population from the liver.
8. VDZ causes an overall rise in ALP in patients with PSC/IBD.

9. There is heterogeneity in clinical response to VDZ in patients with PSC/IBD, where a small subset have improvement of ALP, a moderate subset have ALP rise, and some have no change in ALP.
10. VDZ causes a rise in gut-homing CD4+ T-cells (CCR9/β7+) within the peripheral blood, and reduces trafficking of β7+ T-cells to the colon.
11. These changes in the peripheral blood and colon are more pronounced in PSC/IBD as compared to IBD alone.
12. Patients on VDZ have a higher proportion of CCR9+ T-cells within the liver compared to those not on VDZ, suggesting VDZ promotes homing to the liver of these cells.

Figure 7-1 illustrates these findings graphically.

In making these findings, I achieved the 6 aims set out in section 1.7 in the introduction. However, some of the hypotheses surrounding the role of gut-homing lymphocytes with the livers of patients with PSC have not been proven.

Nevertheless, there are clear differences within the blood, ileum, and liver with regards to T-cell phenotypes between PSC and controls. The reduction of CCR9+ CD4+ T-cells within the peripheral blood and ileum in PSC lent itself to the suggestion that this might be due to trafficking of these cells initially to the inflamed ileum, then aberrant homing to the liver. Experiments analysing LILs in PSC shown in Chapter 4 do not support this. PSC is a heterogenous disease, as demonstrated by the differing natural history of the disease in patients. Some patients develop liver failure, some develop malignancies, some have

active IBD, and some lead a fairly indolent course. It is possible that this hypothesis of aberrant homing to the liver is relevant in a subset of patients with PSC.

The finding of higher proportions of CCR9+ T-cells within the liver (i.e. 5-10% of T-cells) in subsets of patients within the liver control cohort as well as the PSC cohort suggests a role for these cells in chronic inflammation within the liver. I could not find any correlation between CCR9 expression and fibrosis as determined by liver stiffness measurement, but I suspect these cells may play a pro-inflammatory role and home to the liver via upregulation by the sinusoidal endothelium of relevant chemokines and adhesion molecules. It would be interesting to assess patients' clinical status several years down the track to evaluate whether higher proportions of CCR9+ cells (and possibly CD69+ cells) is associated with a poorer clinical outcome.

However, despite the differences found within the ileum of CCR9+ T-cells, I do not believe that gut-homing T-cells are implicated in the hepatic pathophysiology specifically as compared with other liver diseases. They may play a role more generally in inflamed livers, but these data suggest they are not unique to PSC.

These data do, however, show that the IBD in PSC has its own immunological phenotype, and this is the first study to suggest that there are immunological differences which lie within the ileum. The differential effects of VDZ within the colon in PSC/IBD vs. UC suggest some characteristic differences within the colonic endothelium, which may include differing MAdCAM-1 or CCL25 expression, though this has never been examined in PSC-IBD.

These data are concerning for VDZ in PSC/IBD as there was a proportion of patients who had a significant ALP rise and this was associated with the presence of gut-homing T-cells within the liver. It is unlikely that redirection of CCR9+/β7+ T-cells is the case for all patients receiving VDZ, given that there has not been a liver hepatotoxicity signal in UC registration trials of VDZ, and indeed it is beneficial to the IBD in PSC. Whether this re-homing (with VDZ) of T-cells from the peripheral blood and gut to the liver in these patients is truly detrimental to their clinical course is not clear. The clinical data in this thesis implies that a small proportion of patients with PSC/IBD may stand to benefit, particularly those with cirrhosis and those with initially raised ALP (and presumably more inflammatory disease).

I believe VDZ may play a detrimental effect in some patients with PSC, as they may have increased hepatic expression of CCL25 leading to hepatic recruitment of CCR9+ T-cells which have been mobilised within the peripheral blood by blocking their recruitment to the gut. Why they may still enter the liver and not the gut is an interesting question, but it is likely that the mechanisms controlling tissue recruitment of lymphocytes is less tightly regulated within the liver (and in particular the liver sinusoidal endothelium) compared with the gut. This is due to the slower velocity of blood flow within the sinusoids, the fenestration of the LSECs and the upregulation of a variety of chemokines and adhesion molecules in inflamed LSECs. Indeed, α4β7, though prevented from binding to MAdCAM-1, may bind to VCAM-1 which may assist hepatic recruitment of these cells.

Future work needed includes further interrogation of the LILs from patients with PSC and LCs via scRNAseq at a higher resolution to look for further immunological and transcriptional differences, with subsequent validation at the protein level. CCR9-blocking therapies may also be of benefit in PSC in selected patients, though this may also be relevant in patients with other chronic liver diseases, and further translational work in this area is warranted.

In conclusion, I have shown differing distributions of gut-homing lymphocytes, particularly CCR9+ CD4+ T-cells, within the peripheral blood, ileum, and liver in PSC as compared to controls, and have shown the changes which occur with VDZ in PSC/IBD and IBD at a clinical and cellular level. These data do not support the role of gut-homing lymphocytes in the pathophysiology of PSC but these cells may play an important role in chronic inflammatory liver diseases in general. I have further developed the technique of liver FNA for sampling LILs. Future studies utilising this technique serve to greatly enhance our understanding of PSC and other chronic liver diseases.

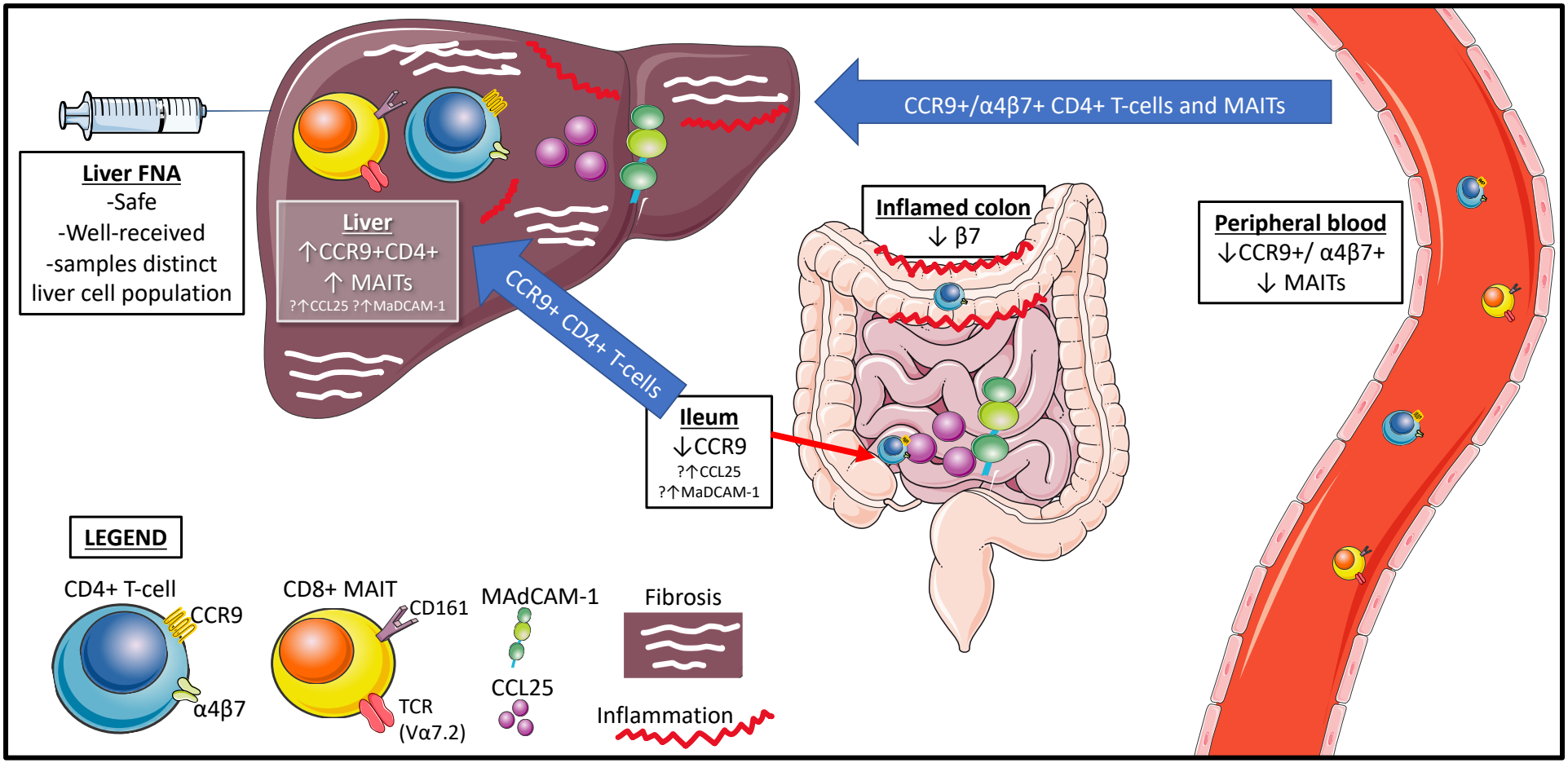


Figure 7-1 - Graphic representation of findings made within the PSC peripheral blood, intestine and liver as compared with controls

The size of the T-cells (CCR9+/α4β7+ CD4 T-cells and MAITs), chemokines (CCL25) and adhesion molecules (MAdCAM-1) indicates the relative proportions of these cells/molecules in PSC as compared with controls. The blue arrows are the suggested homing pathways of certain cells, and question marks denote hypotheses made as to the explanations for some of the findings, but which have not been tested in this thesis. FNA, fine needle aspiration; MAIT, mucosa associated innate-like T-cell; PSC, primary sclerosing cholangitis; TCR, T-cell receptor

8 Appendices

8.1 γAntibody panels for flow cytometry

8.1.1 Panel 1 – examining CCR9+, β7, and CD161++ cells in peripheral blood, intestine, and liver

Antigen	Fluorochrome	Brand	Ref number	Clone	Dilution
CD3	Vio Blue	Miltenyi Biotec	130-094-363	BW264/56	1:100
CD4	Vio Green	Miltenyi Biotec	130-096-900	VIT4	1:100
CD8	PE-Cy7	Miltenyi Biotec	130-096-556	BW135/80	1:100
CD161	PE	Miltenyi Biotec	130-092-677	191B8	1:50
Vα7.2	FITC	BioLegend	351704	3C10	1:50
CCR9	APC	BioLegend	358908	L053E8	1:50
β7	PerCP-Cy5.5	BioLegend	321220	FIB504	2.5:100
Live/dead	Near IR	Life Technologies	L10119		1:300

8.1.2 Panel 2 – looking also at αEβ7 positive cells

As per Panel 1, but substituted Vα7.2-FITC with:

Antigen	Fluorochrome	Brand	Ref number	Clone	Dilution
CD103 (αE)	FITC	BD Pharmingen	550259	Ber-ACT8	1:50

This panel was initially designed so as to exclude any beta7 cells which were also positive for αE, thinking this would gate out αEβ7, leaving only α4β7 cells within the β7 positive population. After trying out the panel, it quickly became apparent that lymphocytes expressed *both* αEβ7 and α4β7 on the same cells, and therefore, gating out the αEβ7 positive cells would remove α4β7 positive cells which needed to be included. This panel was therefore abandoned.

8.1.3 Panel 3 – Incorporating CD45RA and CD69

Antigen	Fluorochrome	Brand	Ref number	Clone	Dilution
CD3	Vio Blue	Miltenyi Biotec	130-094-363	BW264/56	1:100
CD4	VioGreen	Miltenyi Biotec	130-096-900	VIT4	1:100
CD45RA	BV650	Miltenyi Biotec			1:50
CD8	PE-Cy7	Miltenyi Biotec	130-096-556	BW135/80	1:100
CD161	PE	Miltenyi Biotec	130-092-677	191B8	1:50
CD69	FITC	BioLegend	358908		1:50
CCR9	APC	BioLegend	321220	L053E8	1:50
β7	PerCP-Cy5.5	Life Technologies	L10119	FIB504	2.5:100
Live/dead	Near IR	Life Technologies	L10119		1:300

This was a temporary Panel used to incorporate CD69 and CD45RA into the initial Panel 1 for use on the three laser LSR II. There were some compensation issues and CCR9 expression within ileum was very low so this panel was abandoned, and there are no results with this panel in the results section of the thesis.

8.1.4 Panel 4 (and Panel 5)

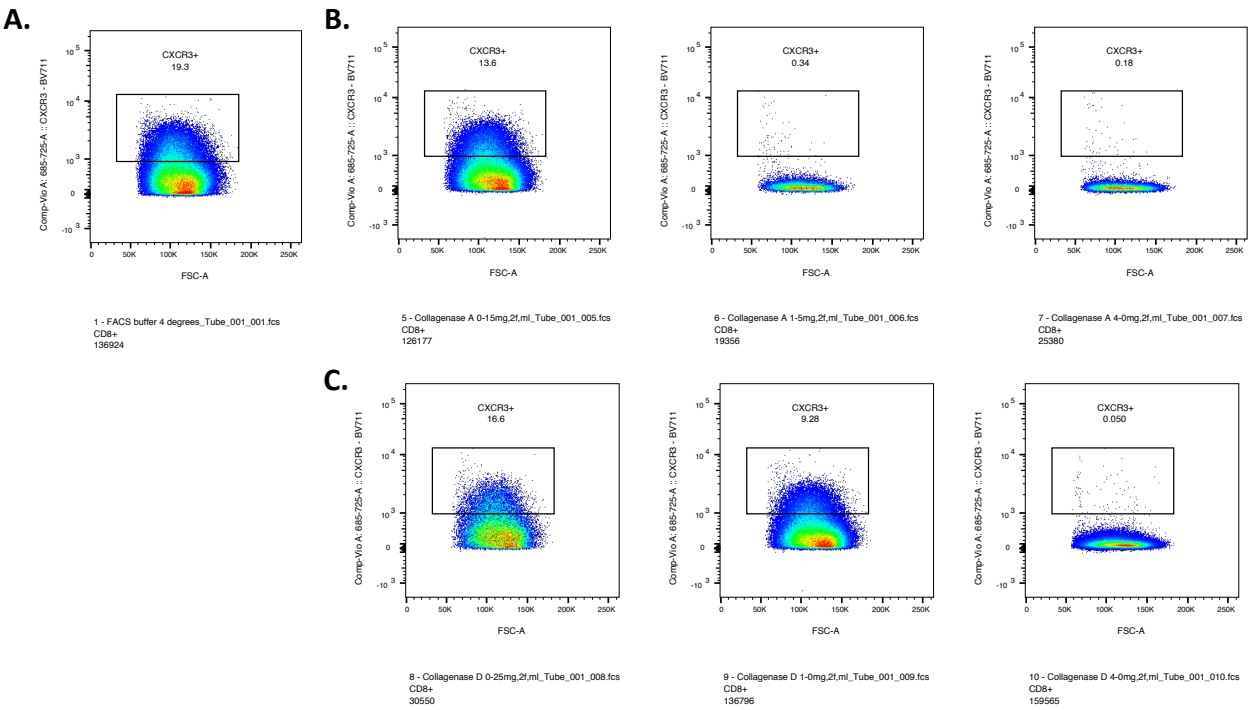
Antigen	Fluorochrome	Brand	Ref number	Clone	Dilution
CD3	BV711	BD Biosciences	300463	UCHT1	1:100
CD4	BV480	BD Biosciences	566165	SK3	1:100
CD8	APC-R700	BD Biosciences	565192	SK1	1:100
CD161	PE-Vio770	Miltenyi	130-099-967	191B8	1:50
Vα7.2	BV605	Biolegend	351719	3C10	1:50
CCR9	APC	Biolegend	358907	L053E8	1:20
β7	PE	Biolegend	321203	FIB504	2.5:100
CXCR3	BV421	BD Biosciences	353715	G025H7	5:100
CD45	FITC	Biolegend	304005	HI30	1:100
CD45RA	PE-Dazzle 594	Biolegend	304145	HI100	2:100
Live/dead	Zombie Near-IR	Biolegend	423105		1:1000

8.1.5 Panel 6 – Incorporating CD56

Antigen	Fluorochrome	Brand	Ref number	Clone	Dilution
CD3	BV785	BD Biosciences	317330	OKT3	1:100
CD 69	BV711	Biolegend		FN50	1:100
CD4	BV480	BD Biosciences	566165	SK3 (aka Leu3a)	1:100
CD8	APC-R700	BD Biosciences	565192	SK1	1:100
CD161	PE-Vio770	Miltenyi	130-099-967/5	191B8	2:100
Vα7.2	BV605		351719	3C10	5:100
CCR9	APC	Biolegend	358907	L053E8	5:100
β7	PE	Biolegend	321203	FIB504	2:100
CD56	BV421	Biolegend	318327/8	HCD56	2:100
CD45	FITC	Biolegend	304005	HI30	1:100
CD45RA	PE-Dazzle 594	Biolegend	304145	HI100	2:100
Live/dead	Near IR	Life Technologies	L10119		0.33:100

8.2 Effect of temperature and collagenases on CCR9 and beta7 expression

Collagenase was required for homogenisation of intestinal tissue in the process of extracting mononuclear cells, but it was not clear whether these enzymes had any effect on chemokine receptors and integrins of interest (such as cleaving them, thereby preventing antibody-mediated recognition of these antigens, and producing a lower percentage than actually present). Traditionally, Collagenase A has been used for intestinal tissue homogenisation and dissociation, but another option was Collagenase D through a protocol devised in the Translational Gastroenterology Unit previously. Therefore peripheral blood mononuclear cells were incubated with differing concentrations of Collagenase A and D, and the proportion of CD4+ and CD8+ T-cells expressing CCR9, Beta7, CXCR3, and CD161++ were compared among conditions. Typical concentrations for cell isolation protocols are 1.5mg/ml for Collagenase A and 1.0mg/ml for Collagenase D. Figure below shows an example set of flow cytometry plots and table below shows the results.



Example of a flow cytometry scatter plot showing effect of varying concentrations of collagenases on chemokine expression among peripheral blood CD8+ T-cells.

A - CXCR3+ expression in CD8+ T-cells incubated at 4°C in cell staining buffer with no added collagenase (i.e. control). B – CXCR3+ expression in T-cells incubated at 37°C with Collagenase A: 0.15mg/ml (left), 1.5mg/ml (middle), 4.0mg/ml (right); C - CXCR3+ expression in T-cells incubated at 37°C with Collagenase D: 0.25mg/ml (left), 1.0mg/ml (middle), 4.0mg/ml (right).

Proportion of peripheral blood T-cells positive for CCR9, β7, CXCR3 and CD161 (++) after incubation under various different conditions.

Antigen	Cell subset	Cell staining buffer		Collagenase A (mg/ml)			Collagenase D (mg/ml)		
		4°C	37°C	0.15	1.5	4.0	0.25	1.0	4.0
CCR9	CD4+	2.98%	3.38%	3.05%	4.85%	3.46%	3.20%	2.85%	2.93%
	CD8+	0.79%	1.25%	0.60%	1.43%	0.97%	1.07%	0.48%	0.57%
β7	CD4+	16.9%	9.53%	17.1%	13.1%	11.6%	17.2%	14.3%	8.26%
	CD8+	4.30%	1.64%	4.05%	2.26%	1.88%	4.23%	3.04%	1.33%
CXCR3	CD4+	33.2%	22.7%	30.8%	2.59%	0.86%	30.8%	27.2%	1.60%
	CD8+	19.3%	9.61%	13.6%	0.34%	0.18%	16.6%	9.28%	0.05%
CD161++	CD4+	1.57%	1.94%	1.20%	1.25%	1.52%	1.08%	1.42%	2.64%
	CD8+	2.50%	3.13%	2.18%	3.71%	4.37%	3.15%	2.48%	3.17%

Cell staining buffer contained no collagenases, with incubation in cell staining buffer at 4°C as the control. All collagenase incubations were done in culture media (cRPMI) in a 37°C shaking incubator for one hour. Where a figure is in red, this represents a greater than 25% drop from control.

Collagenase had little effect on the expression of CCR9 except for a 40% drop among CD8+ T-cells at moderate concentration Collagenase D (1.0mg/ml), and greater still for high concentration Collagenase D. Likewise there was a small drop in CD8+ beta7 expression in both Collagenases moderate concentrations, more pronounced with Collagenase A. Collagenase A dramatically reduced CXCR3+ expression in both CD4+ and CD8+ T-cells, whereas Collagenase D had only a mild-moderate effect on CXCR3+ expression among CD8+ T-cells only. CD161++ expression was not affected by collagenases. Incubating at 37°C caused a reduction in β7 and CXCR3 expression as compared with 4°C incubation.

It was felt therefore, that Collagenase D performed better than Collagenase A in preserving expression of these particular antigens, and this collagenase was therefore used going forward with intestinal mononuclear cell isolation.

8.3 Optimisation of LIL isolation from liver biopsies and resections

Various different methods were trialled to extract mononuclear cells from either liver biopsies or liver resections. Percutaneous liver biopsy was performed under ultrasound guidance with a Quick-core Biopsy needle set with a 2cm stylet (Cook Medical, QC-18-7.0-20T), and various types of liver resections were performed by hepatobiliary surgeons for removal of tumours with a sample taken from the edge of the resection which represented non-tumour liver. In both cases, samples were placed in culture media (RPMI-1640, 10% foetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine [Sigma Aldrich]) on ice. The average weight of a liver biopsy sample was 20-30mg, and for liver resections, the weights varied from 200mg to 1700mg, but only samples up to 80mg were used for extraction.

Initial optimisation of the protocol was carried out with a liver resection specimen. The liver sample was divided into 4 equal samples (40-50mg each) and each was dissected into smaller pieces in chilled culture media in a petri dish. Media was changed several times so as to wash as much peripheral blood from the specimen. Each sample underwent a slightly different process. The various conditions were:

1. **Mechanical digestion** only (100μm filter into a 50ml Falcon™ conical tube (Thermo Fisher Scientific) and pellet derived from centrifugation
2. Incubation with **Collagenase/DNase** (concentrations as per intestinal LPMC isolation) for 1 hour at 37°C, then mechanical digestion
3. Mechanical digestion then run on **Percoll®** (Sigma-Aldrich) gradient (35%/70%)
    a. Cells taken from Percoll® gradient
    b. Cells taken from pellet
4. Mechanical digestion then run on **Lymphoprep™** (Stem Cell Technologies) gradient
    a. Cells taken from Lymphoprep™ gradient
    b. Cells taken from pellet

This resulted in 6 samples, the viable mononuclear cell yield of which were determined by staining with Trypan Blue and counted on a haematocytometer on a microscope, and purity of specimen (e.g. contamination by parenchymal cells) assessed. Yield specifically for CD3+ T-cells, and its subsets CD4+ and CD8+, were then determined using flow cytometry (Antibody Panel 1). Results are in table below.

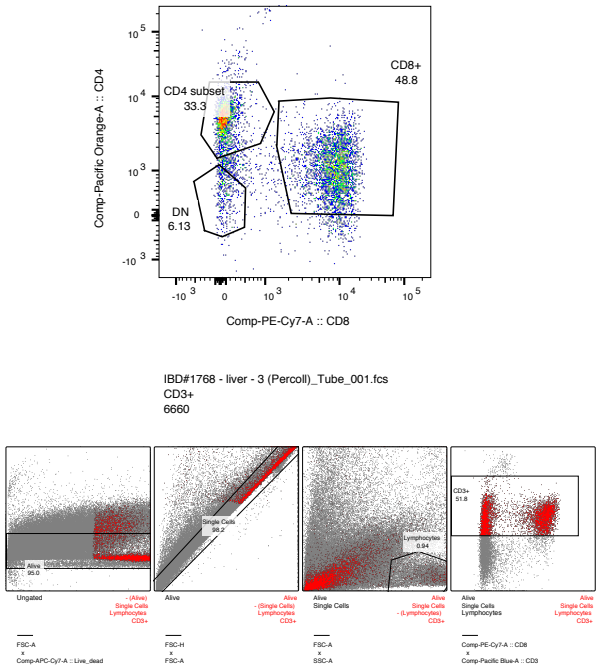
It was apparent that unless the cells ran on a gradient, that there were many non-mononuclear cells retained, suspected to be parenchymal cells such as hepatocytes and biliary epithelial cells, as well as red blood cells. The highest yield as counted on the haematocytometer came from the Percoll gradient strategy and then the mechanical strategy, which was true also for flow cytometry counting. It was felt that given the better purity of cells obtained through the Percoll gradient, this should be the method employed. The scatter plots for the Percoll gradient cells is shown in figure below. In subsequent experiments isolating mononuclear cells from livers, both the Percoll gradient and Percoll pellet cells were isolated and counted and stained, but, as with this initial experiment, very poor yields came from the Percoll pellet.

Therefore, subsequent experiments described in this thesis of liver infiltrating lymphocytes derived from liver biopsies and resections were obtained via the method described above (3a, Mechanical digestion then Percoll gradient).

Cell yields from various methods of isolating mononuclear cells from liver resection.

	Method	Haematocytometer	Count by flow cytometry		
		#mononuclear cells (x1000)	#CD3	#CD4	#CD8
1	Mechanical	630	8795	3016	4534
2	Collagenase/DNase	210	6660	2216	3253
3A	Percoll gradient	1300	9108	2931	4643
3B	Percoll pellet	90	1929	1026	554
4A	Lymphoprep gradient	60	2262	917	1055
4B	Lymphoprep pellet	345	5218	1973	2208

The Percoll gradient method gave the best yield with the purest results.

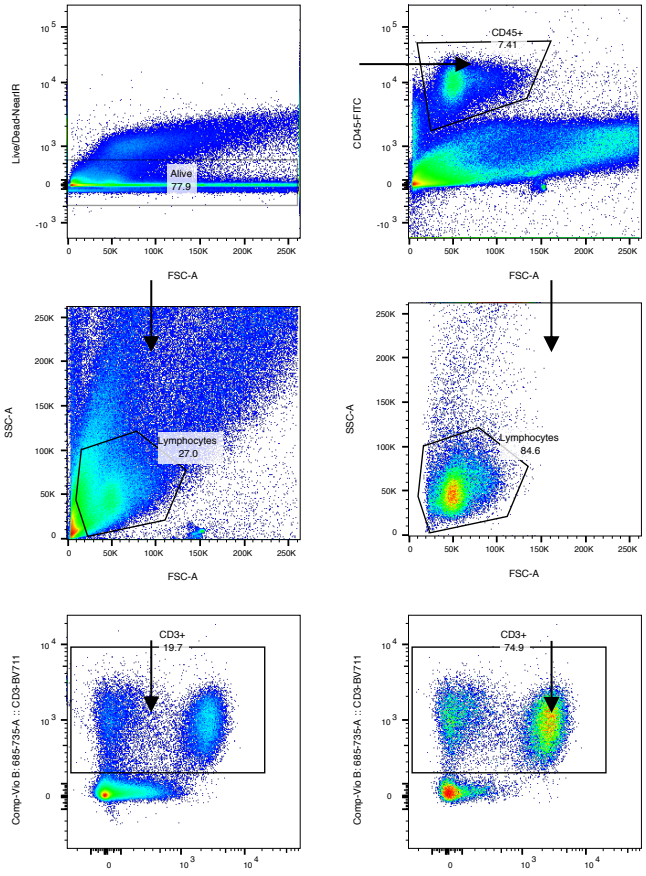


Flow cytometry scatter plot of CD4+ and CD8+ lymphocytes isolated from liver resection sample using a Percoll gradient.

Backgating is shown in the bottom panel.

8.4 Optimisation of a new flow cytometry panel – CD45/CD54RA

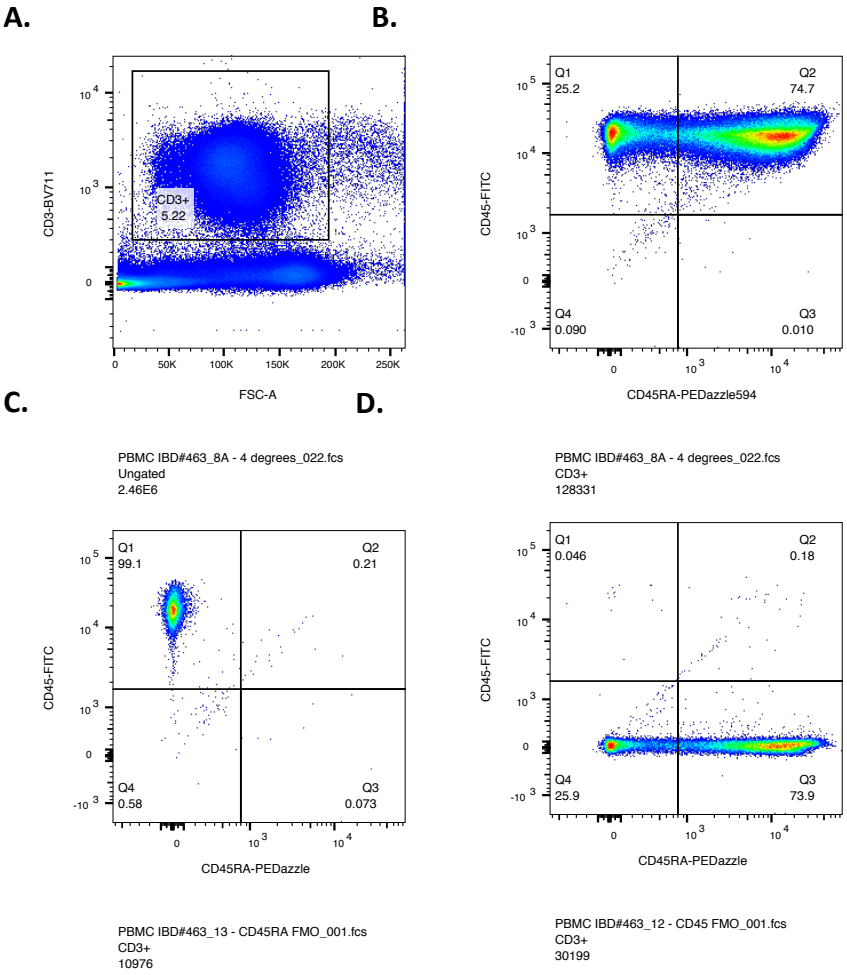
It became apparent that the scatter plots produced from intestinal derived lymphocytes were not very clean, in that they did not clearly show the lymphocyte population, and often backgating had to be employed. By including CD45 staining in the panel, it was felt this would solve this situation to bring tissue scatter plots of FSC-A vs. SSC-A more in line with those of peripheral blood. This is exemplified in figure below.



Flow cytometry scatter plots using CD45 to assist with gating for lymphocytes isolated from ileal tissue.

Arrows show gating strategy. When incorporating gating only on CD45+ cells (top right panel), this leads to a much clearer visualisation of lymphocyte population (middle right) as compared with no CD45+ gating as in earlier panels (middle left). CD3+ populations also become more distinct.

Additionally, it was felt that CD45RA should be included so as to incorporate memory status to findings, but it was not clear whether staining with both CD45 and CD45RA would capture all positive cells for these antigens, or whether there would be competitive binding. A simple experiment staining PBMC with CD3+, CD45 and CD45RA showed that all C45RA+ cells were also CD45+.



Flow cytometry scatter plots demonstrating CD45 and CD45RA expression on CD3+ T-cells in peripheral blood. All CD45RA+ cells are also CD45+, demonstrating that the antibodies used for this flow cytometry experiment do not compete for the same antigen binding site. A – CD3+ gating; B – CD45 and CD45RA gating. C – CD45RA FMO sample; D – CD45 FMO sample.

8.5 Study documents for the PSC FNA Liver Study.

8.5.1 Inclusion and exclusion criteria

Inclusion Criteria:

- Participant is willing and able to give informed consent for participation in the study.
- Male or Female, aged 18 years to 75 years.
- Upper abdominal ultrasound within the last 12 months (or, willing to undergo an upper abdominal ultrasound prior to FNA Liver).

PSC Cohort: extra inclusion criteria

- Diagnosed with Primary Sclerosing Cholangitis according to standard clinical criteria for >3 months.

Control cohort: extra inclusion criteria

- Patients with suspected or definite chronic liver disease.
- Scheduled to have a liver biopsy for clinical purposes during the study period.

Exclusion Criteria:

The participant may not enter the study if ANY of the following apply:

- Unable to consent.
- Significant comorbid medical condition(s) which may increase the risk of an FNA Liver.
- Blood tests within previous 3 months which would increase risk of bleeding:
    - INR>1.3 or PT>16 seconds.
    - Platelet count <100 x 10^3/L.
- Known hepatobiliary malignancy.
- Clinical suspicion or evidence of ascites.
- Use of an oral anticoagulant or antiplatelet agent.
- Pregnancy.
- Any concern by the investigator regarding the safe participation of the patient in the study; or investigator's consideration, for any other reason, that a patient is inappropriate for participation in the study.

8.5.2 Patient information sheet for PSC Cohort

Analysis of liver infiltrating lymphocytes in primary sclerosing cholangitis by fine needle aspiration of the liver.

Patient Information Sheet
PSC Cohort

Chief Investigator: Dr Kate Williamson
Investigators: Prof Satish Keshav, Prof Paul Klenerman, Dr Roger Chapman, Dr Jane Collier, Dr Jeremy Cobbold, Dr Stephanie Slevin, Dr Nicholas Provine

1. Invitation

You are invited to take part in an educational clinical research study carried out by the University of Oxford in conjunction with the Gastroenterology Unit at The John Radcliffe Hospital. Before you decide whether or not you wish to take part, you should read the information provided in this leaflet carefully and if you wish, discuss it with your family, friends or GP. Take time to ask questions – do not feel rushed or under any obligation to make a hasty judgement. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you – this process is known as 'Informed Consent'.

2. Why is this study being done?

Despite encouraging advances in our understanding of primary sclerosing cholangitis (PSC), we still do not fully understand the cause of the disease, nor is there a beneficial medical treatment to slow its progression.
PSC is an immune-mediated disease. Immune cells called "lymphocytes" are thought to play a key role in its development. Lymphocytes that usually reside in the gut are also in the liver of people with PSC but not in the liver of people with other liver diseases. It is thought that the migration of these lymphocytes from the gut to the liver contributes to the development of PSC. However, research to date has focused on lymphocyte activity only in people with advanced PSC.
To address this further, we need to analyse the lymphocytes which are present in the liver in PSC in all stages of the disease, and compare them with liver lymphocytes from people without PSC. The only way to obtain lymphocytes from the liver is to take a sample directly. This may be done through a very low risk procedure called fine needle aspiration with minimal to no side effects to the patient.
A range of experiments will then be carried out on the samples obtained to better understand the mechanisms that lead to the development and progression of PSC. This study is educational in nature and will help us learn more about these causative processes, which could potentially enable us to design and target better therapies for PSC.

3. Why have I been invited?

You have been invited because you have been diagnosed with Primary Sclerosing Cholangitis. We are inviting up to 50 patients with PSC to take part, as well as 50 control patients who do not have PSC (but have some other form of chronic liver disease).

4. Do I have to take part?

No. It is up to you to decide as to whether or not to take part. Not participating will have no effect on your future care.
You may change your mind at any time, before the start of the study or even after you have commenced the study, for whatever reason, without having to justify your decision. This too will have no negative impact on the care you receive.

5. What will happen to me if I decide to take part?

If you decide to take part, we will arrange for you to have a Study Visit with us to obtain samples (detailed below). Prior to that we will check that you have had a recent abdominal ultrasound (in the last year) as well as a recent routine blood test (in the last three months). If you have not had these done recently, we will arrange for you to have these done before taking part in the study. These are part of your routine clinical care, and can be done local to you (e.g. GP surgery) or at the hospital.
We will contact you via phone or email to set up a time for your Screening Visit and Study Visit 1. We want to try and minimise the amount of time you have to come to the hospital, so usually these two visits could occur on the same day (immediately one after the other). But, if you wish, we can organise the Screening Visit on an earlier date, so you can talk through the trial with us in person before entering the study, and thereby leaving some time between entering the study and Study Visit 1.

Screening Visit
This will be a one-on-one consultation with one of the investigators, who is also a medical doctor. You will have an opportunity to ask questions about the study and then if you are happy to participate, you will sign the Informed Consent Form to confirm you have entered the study. The investigator will go through your past medical history with you and check your recent blood tests and ultrasound, and confirm whether you are eligible for the trial. you have not yet had the relevant blood tests and ultrasound, these may be ordered at this stage (blood test can be taken at this appointment).
We expect this Screening Visit would take approximately half an hour, and it would occur at the John Radcliffe Hospital (or, rarely, at an alternative site of Oxford University Hospitals).

Study Visit 1
This may occur straight after the Screening Visit if all the investigations are done, or else on a separate date. At this visit, a sample of blood will be taken – around 25ml, which is approximately 5 teaspoons worth. You will then undergo fine needle aspiration of the liver (further details below), and be observed for an hour afterwards. If you are fine at this stage, you would then be free to go home. We anticipate that Study Visit 1 would

take around 1.5-2 hours in total, though occasionally it may be slightly longer. You will be able to drive and resume work immediately after this visit.

Telephone follow-up 1
You will be called on the telephone one month later to check how you have been since Study Visit 1.
We will also ask you whether you are happy to be contacted at a later date (within one year) to consider having a repeat Study Visit (for another blood sample and a second fine needle aspiration of the liver). This is completely optional, and you will only have to decide about this at the telephone follow up, as by this stage you will have more of an idea of what the study visit (and there for the fine needle aspiration of the liver) entails. If you decline contact with regards to a further Study Visit (which is perfectly fine, and you would not need to give an explanation), then this would be the end of your trial involvement.

Study Visit 2 (optional) – within one year of telephone follow-up 1
This would be exactly the same as Study Visit 1 – a blood sample and fine needle aspiration of the liver with a short observation period afterwards. As mentioned previously, this second study visit is not routine, and would be an optional extra visit, which you only decide if you want to do at the first telephone follow-up.

Telephone follow-up 2 (optional - only if Study Visit 2 has occurred)
You will be called on the telephone one month after Study Visit 2 to check how you have been. This would be the end of your trial involvement.

6. What is involved in a fine needle aspiration of the Liver?

This procedure to obtain cells from the liver has been performed for over 100 years. It originally was designed to take samples to help make a diagnosis of various liver conditions but over the last 12 years, it has been successfully used to obtain lymphocytes from the liver, particularly in order to perform research into specific immune changes associated with specific liver diseases. In over 500 procedures performed for research, no serious complications have occurred. This technique has been used in various liver diseases, particularly Hepatitis C, but never specifically for PSC.
Fine needle aspiration of the liver (FNA liver) is similar to a liver biopsy but is much lower risk as it uses a much finer/thinner needle. You lie down on a bed with your right arm resting above your head. You will be asked to hold your breath out (i.e. exhale) for short amounts of time for some of the following steps of the procedure. An ultrasound would be used to find the best location for the procedure. This is usually between two of your lower ribs on the right-hand side of your upper abdomen.
The area would be cleaned with a sterilising solution and then some local anaesthetic would be injected into the skin and slightly more deeply with a fine needle in this area. This causes a stinging sensation in the area which would last between 10 to 20 seconds. Most patients can tolerate this discomfort fairly well, and the discomfort would then subside.
In the same area, a very fine needle (similar width to a butterfly needle which can sometimes be used when taking blood) will then be inserted through the skin to the edge of the liver. You would not feel any pain from this except for the very rare occasion when

the local anaesthetic has not completely numbed the area in which case minor discomfort is experienced. Using suction, the doctor will aspirate cells into a syringe. This will occur twice (without moving the needle) and will take approximately thirty seconds.
The needle is then removed and a bandage is applied to the area with some firm pressure. The whole procedure from the ultrasound to the bandage going on takes around 10-15 minutes. You will then be observed for one hour lying supine on a bed, and have measurements such as blood pressure and heart rate taken. You will be discharged home after one hour after review by the doctor. There are no restrictions on driving or working following the FNA liver. If there are any concerns post-procedure you may be observed for longer, but this is expected to occur only rarely.

7. What are the possible risks of taking part in the study?

The blood samples that you give during this study involve minimal risk but may be associated with minor discomfort, redness or bruising.
Unlike liver biopsy, which carries a 1 in 100 risk of a serious complication, there have been no serious complications reported in the medical literature for FNA liver. Although you may experience some minor discomfort after the local anaesthetic wears off (typically within 30 minutes), this is usually only very mild. If you are in discomfort you will be offered simple analgesia such as paracetamol and occasionally codeine, but actually patients rarely require any pain relief medication.
Despite the very safe track record of FNA liver, there are theoretically very tiny risks of bleeding within the liver, putting a hole (perforation) in a nearby organ (such as the gallbladder, bowel, or lung), injuring a bile duct in the liver, and significant persistent pain. It must be stressed that these complications have never been reported for FNA liver, but you must be aware of the tiny possibility of these. These complications are usually manifested by severe persistent pain in the right upper quadrant of the abdomen, and appropriate clinical care would be implemented either by the investigators of the study or your treating clinician. This would include imaging of the liver (such as a CT scan), pain relief, and sometimes admission to hospital. Surgery is rarely required for these complications – most of them settle with bedrest and observation.
In order to reduce the chance of any complications, the following precautions will be taken:

- An experienced doctor will carry out the procedure under ultrasound guidance.
- The procedure is performed under sterile conditions to ensure minimal risk of infection.
- Your recent blood test will be checked to assess the thinness (clotting) of your blood. If any problems are found in the blood clotting you will not be entered into the study.
- All patients with a known cancer (which can increase the risk of bleeding) will be excluded.
- All patients who have ascites (fluid in the abdominal compartment normally requiring diuretic tablets) will be excluded.
- If you are pregnant, you will not be entered into the study.

8. What are the possible benefits of taking part?

By taking part in this research you are helping to contribute to our understanding of the cause of PSC, and potentially this will help us identify beneficial treatments. You may not benefit yourself directly as a result of this study, but it is hoped the study will benefit future patients with PSC.

9. Will my General Practitioner (GP) be informed of my participation?

Yes, we will send a letter to your GP letting them know you are taking part, and what the study involves.

10. Will my taking part in the study be kept confidential?

Every effort is made to keep records of personal information confidential and secure. The information that you give will be stored with a unique study code rather than your name, on a secure computer system, which will only be accessible by the few individuals that are specifically named in this study.
Complete confidentiality is paramount to us, the research team. Personal information will be kept on a computer to which access is limited. You would also be allocated a unique study code that would be used for all of the tests done on your samples.
Nearly all the information necessary for the research will be used through your unique study code. The researchers using this information are aware of the importance of security and confidentiality even though they do not have access to personal information such as your name or address. The small numbers of researchers that do have the key to personal details all are aware of the importance for confidentiality and have been trained in the ways that information is kept secure. These files containing personal details will be destroyed within 3 years of the study ending. We will endeavor to send you a summary of the study findings within this time period.
Responsible members of the University of Oxford and/or Oxford University Hospitals NHS Foundation Trust may be given access to data for monitoring and/or audit of the study to ensure that the research is complying with applicable regulations.

11. What will happen to the samples I give?

If the samples reveal anything relevant to your care this will be communicated to your usual treating doctor so that they may discuss this with you.
Any samples collected from you will be labelled with your unique Study ID and contain no personal identifiable information. Most of these samples will be used directly in experiments in secure laboratories within the University of Oxford. If you agree, a small proportion of your samples will be stored anonymously within the University of Oxford laboratories to be used in investigations at a later date. Any unused samples will be destroyed after 5 years.
Your anonymised samples will be used mainly by local researchers at the University of Oxford, but ethically approved research projects may take place in hospitals, universities,

non-profit institutions or commercial laboratories worldwide. Any studies carried out on your samples will have appropriate ethical approval.
Some experiments may involve extracting DNA from your sample. The material given to researchers will not have information that identifies you. However, your DNA is unique to you so it can never be completely anonymous.

12. What happens if I don't want to carry on with the study?

Participation in this study is voluntary and you may change your mind at any time. If this occurs, simply contact us via the details given at the end of this leaflet, and let us know you wish to be withdrawn from the study. You do not have to give a reason, and this will not affect your ongoing clinical care.
If you have undergone a FNA liver, we will still phone you at one month to check you are okay for safety reasons, even if you have withdrawn. We may still use the stored samples and/or the data derived from your samples unless you ask us not to, in which case any existing samples or data related to you will be destroyed.
Please note that it may not be possible in all cases, particularly after much time has passed, to destroy or remove samples such as tissue samples or cell lines derived from these, or data associated with these samples, for instance if these have been transferred elsewhere, or used to generate published data.

13. What if there is a problem?

The University of Oxford, as Sponsor, has appropriate insurance in place in the unlikely event that you suffer any harm as a direct consequence of your participation in this study. If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact Dr Kate Williamson (contact details below) or you may contact the University of Oxford Clinical Trials and Research Governance (CTRG) office on 01865 572224, or the head of CTRG, email ctrg@admin.ox.ac.uk.
The Patient Advisory Liaison Service (PALS) is a confidential NHS service that can provide you with support for any complaints or queries you may have regarding the care you receive as an NHS patient. PALS is unable to provide information about this research study. If you wish to contact the PALS team please contact ph: 01865 221473 or email: PALSJR@ouh.nhs.uk.

14. Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect participants' interests. This study has been reviewed and given a favourable opinion by the South Central – Oxford B Research Ethics Committee. In addition, the protocol and study documents have been contributed to and reviewed by the patient charity, PSC Support, and they have formally endorsed this study.

15. Who is organising and funding the study?

The study is sponsored by the University of Oxford and the funding is supplied by an educational grant from the Wellcome Trust. It is being organised by the John Radcliffe Hospital hepatology team and the Keshav and Klenerman laboratory groups in the Translational Gastroenterology Unit.

16. Will I be reimbursed for taking part?

You will be suitably reimbursed for any travel costs incurred for your study visit(s) to the hospital. Otherwise your samples are donated as a gift to the study, and no financial reward will be given for these.

17. What will happen to the results of this study?

The results of this research study may be presented at local, national and international medical and scientific meetings or in publications, and to patient support groups. Some of the research being undertaken will also contribute to the fulfilment of an educational requirement (a doctoral thesis). You will not be identified in any way in publications or reports.
We will list any academic publications derived from this work on the Translational Gastroenterology Unit website available at: http://www.expmedndm.ox.ac.uk/tgu/home.
In addition, a lay report summarising the main results of the study will be sent to you at completion of the study, and will also be published on appropriate departmental and patient websites. You may contact us after you have finished the study to ask about when and where this will be available via the contact information below.

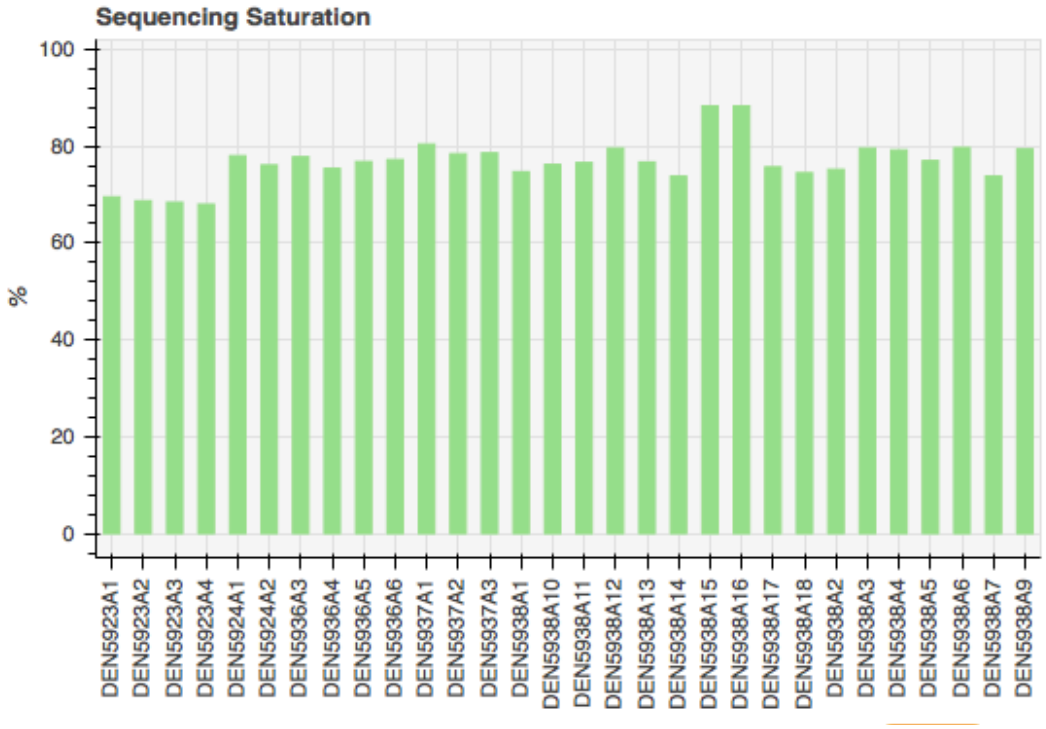
18. Further information and contact details:

For additional information or queries now or at any future time please contact:
Dr Kate Williamson
Gastroenterology Unit, John Radcliffe Hospital
Headley Way, Oxford, OX3 9DU
Email: Kate.williamson@ndm.ox.ac.uk
Telephone: 01865 227 752 (office hours) or 01865 221185 (out of hours, or emergency)

Thank you for considering taking part in this study. If you are interested in taking part, please contact us via the details given above.

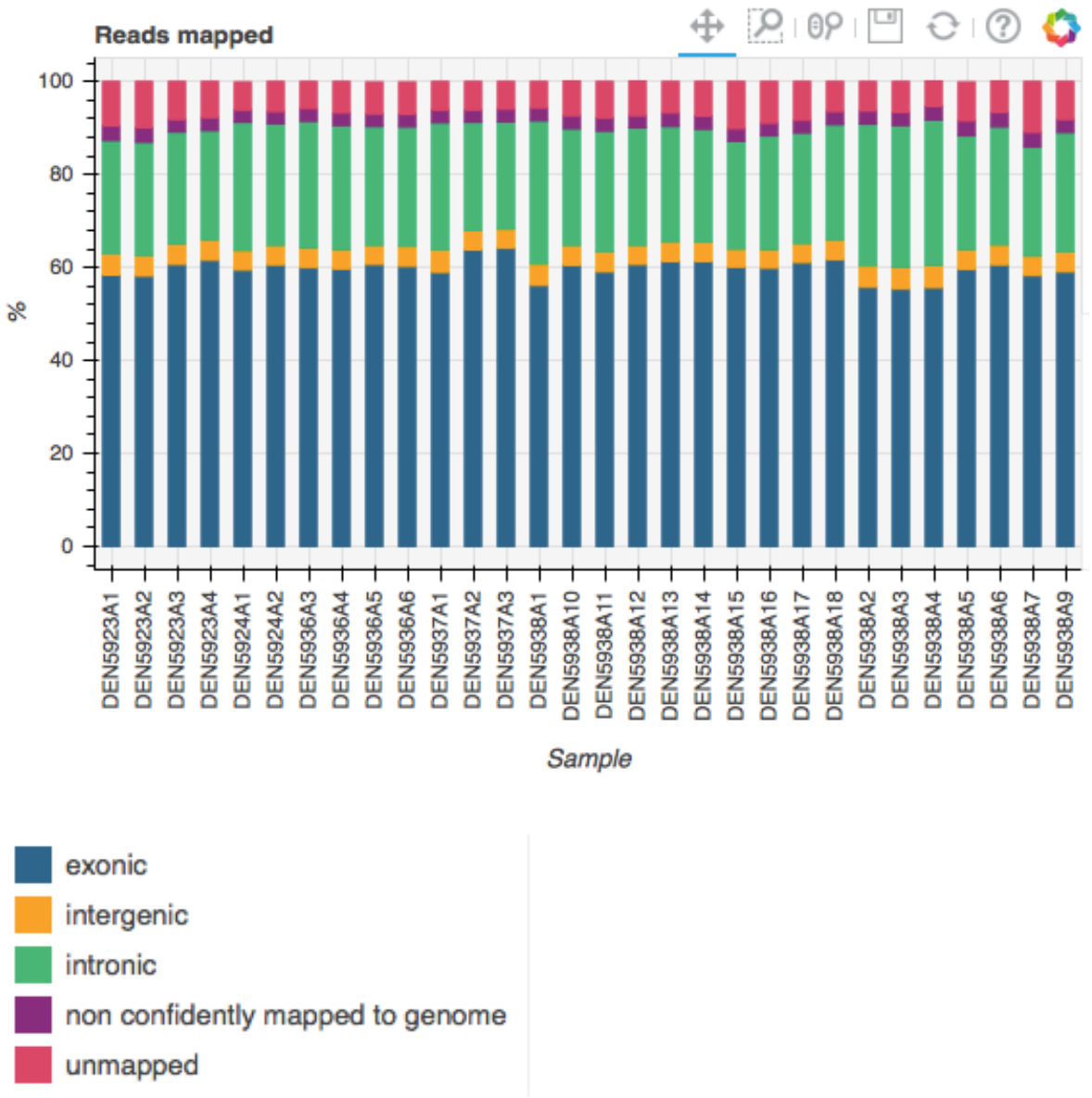
8.6 Quality control for sequencing of FNA and PBMC samples

8.6.1 Sequencing saturation



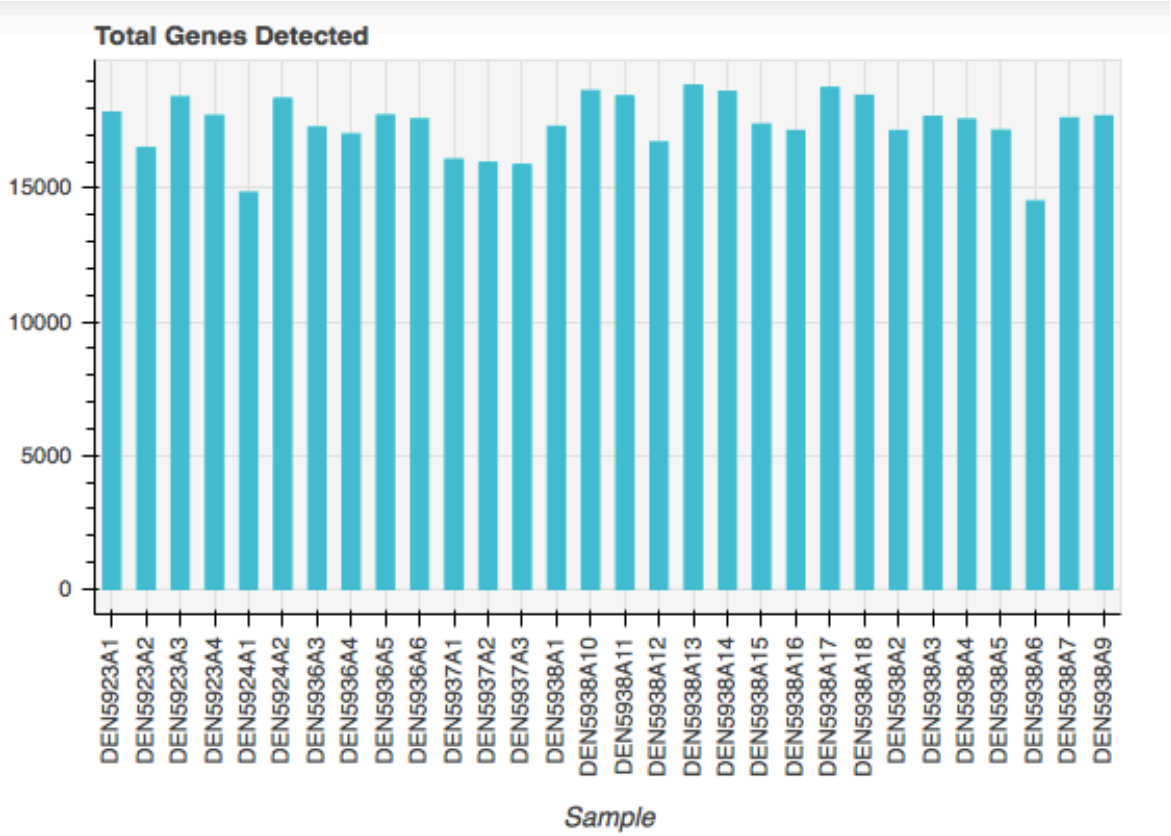
The sequencing saturation was determined for each sample/library which was sequenced. The samples shown here represent the 30 samples sequenced. Whilst there were 9 unique FNA samples and 9 paired unique PBMC, some samples were split over two lines of sequencing. The sequencing saturation is the fraction of reads originating from an already observed unique molecular identifiers (UMI). This is a function of library complexity and sequencing depth. The median saturation was around 80% which is consistent with a reasonable result.

8.6.2 Reads mapped per sample



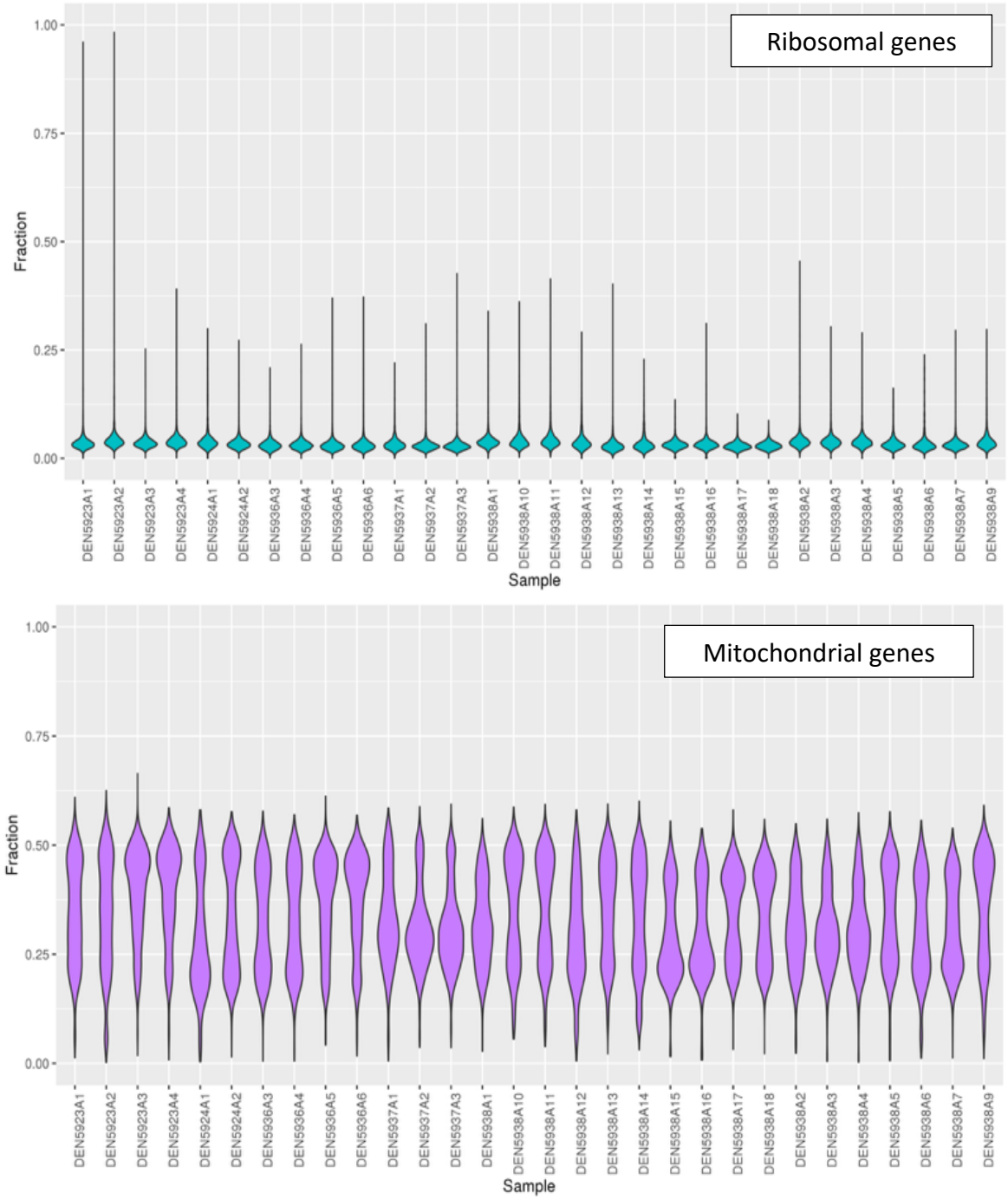
Reads of genes for each sample are classified as exonic, intronic, intergenic, non-confidently mapped to the genome, or unmapped. The types of reads was consistent among all samples as shown in this graph, with typical proportions displayed for sequencing experiments.

8.6.3 Total genes detected per sample



The total genes detected per sample is the number of genes with at least one UMI count in any cell.

8.6.4 Percentage of mitochondrial and ribosomal genes detected



In any sequencing experiment, one aims for low proportion of ribosomal and mitochondrial genes among the genes read. This is because these genes are upregulated in states of cell stress and poor cell viability. In all of our samples there were very low proportions of ribosomal and mitochondrial gene reads, indicating that the samples were viable.

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