Myeloid derived suppressor cells in patients with Pancreatic Adenocarcinoma

A Thesis submitted to the University of Manchester for the Degree of

Master of Philosophy

Faculty of Medical & Human Sciences

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School of Medicine
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Word count: 25,889
Background: Pancreatic cancer (PC) often presents late with poor survival. While the role of immune suppressive cells in pre-clinical studies has generated immunotherapeutic agents, these cells remain under investigated in PC. Myeloid derived suppressor cells (MDSC) are heterogeneous immature myeloid cells that negatively regulate the immune responses during tumour progression, inflammation, and infection.

Aim: The aim of this study was to characterise the different subsets of MDSCs and evaluate their levels and functions in the circulation and tissue of PC patients.

Methods: Flow cytometric staining was performed on peripheral blood samples of 24 PC patients, 12 patients with chronic pancreatitis (CP) and 16 healthy donors. Tumour (n=7) and benign (n=7) pancreatic tissue samples were also examined for comparative analysis.

Results: Significant increases in circulating and tumour-infiltrating granulocytic (Lin-HLA-DR-CD33+CD11b+CD15+), but not monocyctic (Lin-HLA-DR-CD14+), MDSC were observed in patients with PC when compared with healthy donors and age-matched patients with benign pancreatic disease (CP). The circulating MDSCs from PC patients expressed arginase 1 (ARG 1), which represents their functional state. Blood levels of MDSC showed no association with PC stage or preoperative levels of tumour markers.

Conclusions: Our findings provide a first characterisation of the phenotype of different subsets of peripheral and local MDSCs in PC patients and suggest that the frequency and contribution of these cells are predominantly granulocytic. These findings suggest that MDSCs have a role in pancreatic cancer. Future large validation studies may help the development of new immunotherapeutic strategies to inhibit and eliminate MDSCs in PC.
THE UNIVERSITY OF MANCHESTER

LAY ABSTRACT

ABSTRACT OF THESIS submitted by Yazan Sulaiman Khaled for the degree of Master of Philosophy and entitled “Myeloid derived suppressor cells in patients with Pancreatic Adenocarcinoma”.

Month and Year of Submission Submitted June 2014.

It is known that the body’s immune system is important in the recognition and progression of cancer. The immune system protects the body by identifying and destroying viruses, bacteria and body cells that are potentially cancerous. Cancer cells send out chemical signals that dampen down the immune system and consequently allow tumours to grow. Cells have been identified within the immune system that controls the destructive activity in the human body. One of these cells is known as myeloid derived suppressor cells (MDSC). We conducted a study to investigate the levels of MDSCs and their function in the blood system and within the pancreas tissue in patients who suffer from pancreatic cancer (PC). We found that the levels of MDSCs are remarkably increased in the blood and within the PC tissue when compared to normal healthy individuals. We also identified their functional state which suggests a strong correlation with immune suppression in pancreatic cancer. These findings give a better understanding of the role of MDSCs and will help to evolve new treatments for pancreatic cancer in the future.
Declaration

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Dedication

I am indebted to my mother Khadije and father Naief, for their continuous sincere support, invaluable advice without which I would not have reached this stage. I would like to express my gratitude to my daughters (Khadejah & Maryam) and my precious wife Hayat for her encouragement and patience while I have been completing this research. I am also grateful to my brothers (Ahmad & Sari) and sister (Rubie), for their heartfelt thoughts and encouragement. I dedicate this work to the courageous Syrian people in honour of their struggle for freedom.
Acknowledgement

Initially I would like to thank my two supervisors Professor Basil J. Ammori and Dr Eyad Elkord for all their incalculable support and personal as well as critical guidance. I wish to express my appreciation to them for their help throughout the completion of this project.

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I would especially like to thank the patients for their participation in the research project, because without them willingly giving up their time and samples, there would be no project.

The relevant Local Ethics Committee and research and Development Department approved all the research reported in this thesis.
Author thesis based publications:

Peer reviewed journals:


International conference papers:

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Chapter 1
Chapter 1: Introduction and background

1.1 Cancer and Immune system

Cancer is the second leading cause of morbidity and mortality in Europe with 3 million new cases and 1.7 million deaths each year according to the World Health Organisation (WHO). Surgery, radiotherapy and chemotherapy, alone or in combination, have been effective at treating the primary source of the cancer but they failed to control and reduce the metastatic features of cancer [1]. Consequently, the need to develop a new cancer therapy became a must, and many scientists and physicians focused their research on the immune system. The immune system is defined as a highly specialised network of characteristically and functionally diverse immune cells that ultimately protects the host from diverse types of insults such as bacteria, viruses, parasites, cancer cells and maintains an equilibrium between self and foreign antigens [2]. Failure of the immune system to identify, destroy and eradicate the cancer cells has been continuously observed in cancer patients. While tumour infiltrating lymphocytes (TILs) and antigen presenting cells (APCs) were shown to be non-functional within tumour tissue samples, decreased numbers of mature dendritic cells (DCs) were also found in the peripheral blood of cancer patients [2-5]. Another study showed that the antigen-specific CD4 (+) T-cells were rendered unresponsive to the growth of solid tumours indicating that the T-lymphocyte compartment can also be rendered dysfunctional by cancer cells [2, 6]. Tumour-induced immunosuppression is now recognised as a key element through which tumours escape the immune system recognition. Therefore, further investigations into how solid cancers evolve in an immune-competent
environment, evade immune recognition with ultimate progression to a metastatic disease are prerequisites to effectively improve the immune response against cancers.

Cancer immunotherapy, as a new treatment for cancer, has developed remarkably over the past decade to prevent the dissemination of the disease and improve the quality of life of patients [2]. The current approaches of immunotherapy aim to stimulate the immune system to further enhance the immune surveillance of the host to destroy abnormal/cancerous cells and prevent cancer development. Immunotherapy has its clinical applications to treat different cancers. While the administration of cytokines such as interleukin-2 (IL-2) and alpha interferon (IFN-α) has been clinically approved to treat Kaposi’s sarcoma and different type of leukaemias [7, 8], Rituximab, Cetuximab and Trastuzumab are three examples of antibody-based immunotherapy used clinically for the treatment of leukaemia, non-Hodgkin’s lymphoma, breast and colorectal cancers (CRC) [1, 2]. Vaccination against cancers is the latest application of active immunotherapy in cancer treatment and prevention. Tumour antigens are processed by APCs and presented to T-cells which intend to induce an endogenous, long-lasting tumour antigen-specific immune response [1, 2]. Protein-containing vaccines are usually combined with strong adjuvant (BCG) [9] or cytokines (IL-2, IL-12) [10, 11] to induce strong immune responses. DC-based vaccines have also shown promising results in animal models and are under intensive evaluation for clinical applications [2, 12, 13]. Researchers have been successful at activating autologous DC in vitro in sufficient quantities and re-inject them into cancer patients with positive results [14-16]. In a recent multicentre
analysis of the mean survival related to add-on dendritic cell vaccines on patients with inoperable pancreatic cancer receiving chemotherapy, it was reported that The mean survival time from diagnosis was 16.5 months (95% CI 14.4-18.5) and that from the first vaccination was 9.9 months (95% CI 8.0-12.9) [2, 16]. However, this approach is extremely costly and is not feasible for large-scale treatment yet.

Despite these developments in immunotherapy, the effectiveness of cancer vaccines has been hampered by the cellular effect of a group of immune suppressive cells known as myeloid-derived suppressive cells (MDSC) and T regulatory cells (Tregs). They are thought to be largely responsible for inhibiting host T-cell activity against tumour-associated antigens (TAA) and consequently impair the effectiveness of anticancer immunotherapeutic approaches up taking the antigen delivered by vaccination, presenting it to activated T cells, hence inhibiting the same antigen-specific T cells that the vaccination strategy is aiming to activate [2, 17]. Reducing the deleterious effects of these immunosuppressive cells may increase the success of various immunotherapeutic modalities in cancer and improves the efficacy of immunotherapy in both animal models and cancer patients [2, 18]. In a recent in vitro and in vivo study, targeted depletion of granulocytic MDSC in autochthonous pancreatic adenocarcinoma (PADC) was found to increase the intratumoral accumulation of activated CD8 T cells and accelerate the apoptotic rate of tumour epithelial cells suggesting a potential therapeutic strategy for pancreatic cancer (PC) [19]. However, a better understanding of the local tumour microenvironment and the exact mechanisms of MDSC induction and/or expansion in peripheral blood and tumour microenvironment
of cancer patients will provide opportunities to test different treatments to target these immunosuppressive cells and alter the balance in favour of generating more effective anti-tumour immune responses. This could also be vital for the design of more effective immunotherapeutic protocols [2].

1.2 Pancreatic cancer and immunosuppressive cells

Immunotherapy is an attractive modality for treating different cancers including pancreatic carcinoma that should be further explored and developed. However, understanding how immune cells and tumour interact would be critical for immunotherapeutic approaches to succeed.

Pancreatic adenocarcinoma affects 1 in 10,000 populations and carries a poor prognosis [20]. The disease often presents with an advanced stage, and only 10-15% of patients are resectable [21]. The 5-year survival rate for patients with operable disease is estimated to be 15-40% [22].

Similar to other cancer types, Treg levels are increased in pancreatic ductal adenocarcinoma, which is associated with poor prognosis and reduced survival [23]. The relationship between chronic inflammation and cancer has been recognized for centuries, while at least 15–20% of cancers are associated with inflammation [24]. Chronic pancreatitis predisposes to pancreatic carcinoma [2, 25, 26]. The pro-inflammatory IL-17-producing T helper cells (Th17) are a new subset of T cells that have well-described roles in mediating inflammation of autoimmune diseases. Recent studies have suggested a close relationship between CD4+FoxP3+ Tregs and Th17 cells [27]. They share a common lineage and differentiate based on the presence of certain cytokines in the microenvironment; IL-6 in the presence of TGF-β
inhibits Treg development and induces Th17 cells. Recent evidences suggest that Th17 cells are also involved in tumour immunity and may be a target for cancer immunotherapy [2, 28]. Induction of Th17 cells in the tumour microenvironment improves survival in a murine model of pancreatic cancer [29, 30]. There is no available data on the role of MDSC in patients with pancreatic cancer. In mice with spontaneous pancreatic carcinoma, an increase in the levels of MDSC early in tumour development was detected in lymph nodes, blood and pancreas of mice with premalignant lesions and increased further upon tumour progression [2, 31].

1.3 *Origin and definition of MDSC*

Myeloid cells represent the most abundant type of haematopoietic cells in the immune system and have a huge diversity of physiological and pathological functions [32]. The myeloid immature cells are contained within a network of cells that can differentiate into three mature cell types: macrophages, DCs and granulocytes all of which play an important role in the immune system [2, 32]. In cancer and other pathological conditions such as trauma and sepsis, this normal physiological differentiation pathway is partially blocked to generate pathological myeloid cells and an expansion in this population of cells with immunosuppressive functions, hence the term myeloid-derived suppressive cells [33]. Therefore, MDSC are best defined as a heterogeneous population of activated immature myeloid cells characterised by a morphological mixture of granulocytic and monocytic cells but lacks the expression of cell-surface markers that are specific to the fully differentiated monocytes, macrophages or DCs [2, 34]. However, the heterogeneity of the
MDSCs can also be defined according to their expression of cell surface markers as shown below.

1.4 Mice MDSCs

MDSCs are defined in mice by the characteristic co-expression of the myeloid lineage differentiation antigen Gr1 and CD11b (also known as Ly6G/Ly6C and α M-integrin respectively) [35-37]. Granulocytic MDSCs have a CD11b⁺Ly6G⁺Ly6Clow phenotype, whereas MDSCs with monocytic morphology are CD11b⁺Ly6G⁻Ly6C[34, 38-41]. The phenotypical and functional features for both subsets are outlined in Table 1 [2]. The exact role of both MDSC subpopulations in pathological conditions are not fully understood, yet emerging evidence indicates that they have different functions in cancer and autoimmune diseases [2, 38, 42]. In their analysis of ten different experimental tumour models, Youn et al reported that both MDSC subsets were expanded and, in keeping with other studies, the expansion of the granulocytic MDSC population was reported to be much greater than that of the monocytic subset [34]. However, their findings suggested that the level of MDSC expansion is not indicative of their suppressive features but rather a representation of it is functional state within the tumour environment [2, 34]. In more recent studies that aimed to characterise the nature of the granulocytic MDSCs in a murine tumour model, the data showed that although tumour granulocytic MDSCs shared common phenotype cell markers with normal mature neutrophils, granulocytic MDSCs were functionally different [41, 43]. In the last decade, further investigations were aimed at identifying several other functional surface markers of suppressive features of MDSCs such as CD80
Although MDSCs may co-express these surface molecules, their function in MDSC-mediated immune suppression remains unclear. Youn et al observed that MDSCs in tumour bearing mice versus Gr1\(^+\)CD11b\(^+\) subsets from healthy mice co-expressed similar levels of CD115 and CD124 markers and when compared to CD115\(^-\) and CD124\(^-\) subsets they both showed similar ability to suppress T-cell proliferation [2, 34]. Therefore, further investigations are needed to further our understanding of the role and function of these surface markers in the tumour microenvironment [2].

### 1.5 Human MDSCs

The equivalent phenotype of MDSCs in human is traditionally defined as CD14\(^-\)CD11b\(^+\)CD33\(^+\)CD15\(^+\) cells or cells that express the CD33 marker but lack the expression of markers of mature myeloid and lymphoid cells and the MHC class-II molecule HLA-DR [45-47]. This human MDSC phenotype accounts for \(~0.5\%\) of peripheral blood mononuclear cells in healthy individuals [48] with a ten times higher level in the peripheral circulation in patients with different types of cancer such as renal cell carcinoma (RCC) and colorectal carcinoma (CRC) [2, 18, 45, 46, 49]. The identification and isolation of human MDSC subsets has been very challenging due to the heterogeneous characteristics of these immature cells. However, as the characteristic features of MDSC subsets were further examined and identified in different types of human cancers, it has been suggested to re-define the MDSCs by a combination of a new set of functional cell markers such as CD66b and low expression of CD15 [2, 50-52]. Table 2 outlines the different MDSC subsets identified in different human cancers and their
heterogeneous characteristics [2]. These data suggest a significant diversity in the MDSC subsets identified in different human cancers. Gros et al defined the morphology of different MDSC subset as CD14\(^+\) monocytes/macrophages, CD14\(^-\)CD15\(^-\) immature myeloid cells and CD14\(^-\)CD15\(^{hi}\) neutrophils/eosinophils [53]. However, it remains unknown whether this diversity in MDSC subsets is due to different mechanism of induction and/or expansion in different cancers or due to the different surface markers used by the investigators [2]. Nevertheless, more studies are needed to better characterise the phenotypes and functions of human MDSCs and correlate these findings with clinical progression of cancers. In the meantime, MDSCs remain a group of phenotypically and functionally heterogeneous subset of immature myeloid cells with anti-tumour immunosuppressive activity [2].

1.5.1 Expansion and activation of MDSCs in cancer

Accumulating evidence from tumour bearing mice and human cancers indicate that the induction and expansion of MDSCs in tumour microenvironment is mediated by the combined effect of multiple factors including cytokines, growth factors and pro-inflammatory mediators [2, 48]. The list of such factors is constantly growing due to recent laboratory investigations but potential implicated factors in human cancers are divided into two groups [48], ‘MDSC expansion promoting factors’ and ‘MDSC activating factors’. A list of these factors and their signalling pathways is shown in Table 3 [2]. MDSC expansion is facilitated by triggering a cascade through the signalling molecules that regulate cell survival, proliferation, differentiation and apoptosis known as Janus tyrosine kinase (JAK) protein
family members and signal transducer and activator of transcription 3; the main transcription factor involved in MDSCs expansion (STAT3) [48]. In a murine model, pancreatic cancer microenvironment was found to transform monocytes to monocytic MDSCs via STAT3 activation, and hence increase the frequency of Aldehyde dehydrogenase-1 activity (ALDH1) cancer stem cells; a population of tumour cells that are responsible for human PC initiation [54]. In keeping with these findings, Mace et al reported that pancreatic cancer-associated stellate cells (PSCs) promote differentiation of MDSC in a STAT3-dependent manner [55]. PSCs are defined as a subset of pancreatic cancer-associated fibroblasts that provide signals to enhance MDSC differentiation and immunosuppressive function and hence tumour survival. In their analysis of primary PSC cell lines from human samples \((n=7)\), PBMCs were promoted to differentiate into CD11b+CD33+ and CD11b+CD33+CD15+ MDSCs phenotypes which suppressed autologous T-lymphocyte proliferation. This effect was mediated by MDSC-promoting cytokines including IL-6, VEGF, M-CSF, serum-derived factor-1 (SDF-1) and chemotactic protein-1(MCP-1) [55]. In addition, inhibition of STAT3 phosphorylation using FLLL32 STAT3 inhibitor eliminated PSC mediated MDSC differentiation indicating that STAT3 is as a key player for enhancing MDSC immunosuppression in human PC [55].

Increased levels of STAT3 activation were observed in tumour bearing mice, and when myeloid progenitor cells were exposed to a conditioned tumour medium \textit{in vitro}, there was a significant activation of JAK2 and STAT3 with subsequent MDSC expansion [2, 56]. In a most recent study in a melanoma mouse model, the inhibition of tumour-expressed inducible nitric oxide
synthase (iNOS) with a small molecule inhibitor (L-NIL) was associated with an inhibition of accumulation of STAT3/reactive oxygen species (ROS)-expressing MDSCs and an elimination of their suppressive function within the tumour microenvironment [57]. Additionally, normalised serum levels of vascular endothelial growth factor (VEGF) levels were associated with down regulation of the activated STAT3 and ROS production in MDSC and reversed tumour-mediated immunosuppression [2, 57]. In another supporting study, Qu et al reported that inhibition of STAT3 eliminated the suppressive activity of MDSCs in mice [58]. Recent findings indicate that pathways downstream of STAT3 may also regulate MDSCs expansion by inducing the expression of S100A8 and S100A9 proteins; these are members of the S100 calcium-binding protein family that are released in response to cell damage, infection or inflammation and that are involved in the accumulation and function of granulocytic MDSCs specifically [59]. Myeloid progenitor cells expressing S100A8 and S100A9 were found to infiltrate areas of dysplasia and adenoma in both human CRC tissue and colon tumour bearing mice [2, 60]. In addition, these proteins are involved in the production of ROS in MDSCs and in tumour bearing mice model; the absence of S100A9 was associated with an inhibition of MDSC accumulation in the spleen [59]. Although S100A8 and S100A9 induce their effect on MDSCs through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [59], this exact mechanism is still not fully understood but seems to play an important role in regulating MDSC expansion and may provide an interesting link between immune suppression and cancer progression [2].
MicroRNAs (miRNA) molecules have attracted great attention as crucial members of a complex regulatory network that interact to mediate the induction and expansion of MDSCs [2]. miRNAs are small molecules that consist of ~22 nucleotides and have the potential to integrate with transcription factors during the differentiation of MDSCs, modulate the protein gene expression by targeting mRNA during degradation and cause translation disruption [61, 62]. While miR-155, miR-223 and miR-146a may be associated with a cancer microenvironment formation [63, 64], miR-146a plays a negative regulatory role in the development of myeloid cells [2]. Zhao et al reported that the deletion of miR-146a in mice results in a myeloproliferative disorder with an enormous accumulation of MDSCs in the peripheral lymphoid organs [65]. Nonetheless, our understanding of the exact role and function of these molecules and their mechanism of action is very limited and given the preliminary data accumulated from previous studies, further investigations are needed to ascertain whether miRNAs can influence MDSC expansion and/or expansion in human cancers [2].

Accumulating evidence suggests that the immunosuppressive activity of MDSCs is not only dependent on expansion of promoting factors but also on activating factors [48]. The activating factors exert their effect through multiple signalling pathways including STAT6, STAT1, and NF-κB. STAT1 is considered as the major transcription factor, activated by IFNγ and is mainly responsible for the upregulation of ARG1 and iNOS expression in MDSCs within the tumour microenvironment [2, 66]. In mice, it was found that STAT1-negative MDSCs failed to upregulate ARG1 and iNOS expression and subsequently had no inhibitory effect on T cells [66]. The role of STAT6
and its associated IL-4 receptor α-chain (IL-4Rα) in MDSC activation has also been shown in different studies. When MDSCs or cloned MDSC lines were co-cultured with IL-4 in vitro, ARG1 expression was remarkably induced as a marker of activated MDSCs [67]. Although some researchers supported the role of IL-4Rα–STAT6 pathway in facilitating decreased immune surveillance in tumour bearing mice such as sarcoma via the blockage of the ARG1 production [68], others reported that IL-4Rα deficient mice with breast cancer retained a high level of activated MDSC cells after surgical resection [2, 69]. These contradicting studies may indicate that IL-4Rα–STAT6 pathway may not be involved in immunosuppression in all tumour models and that further research is required to identify factors within the tumour microenvironment that regulate this pathway [2].

### 1.6 Mechanisms of MDSC immunosuppression

MDSCs mediate their tumour-induced immunosuppression via several potential mechanisms. It has been shown that MDSCs mediate their suppression of T-lymphocytes in cancer through a direct contact and/or through a combination of multiple major mediators such as iNOS, ARG1, cyclooxygenase 2 (COX-2), prostaglandin E2 (PGE2), TGF-β, IL-10 and Tregs [44, 67, 70-76]. Here we present a summary of the mechanisms of action of these mediators as supported by recent work [2].

#### 1.6.1 Arginase, iNOS and ROS

L-arginine metabolism has been recognised as a major key player in the suppressive activity of the MDSCs (monocytic subset particularly). Arginase (ARG1) and inducible nitric oxide synthase (iNOS) are two different but
related enzymes, expressed highly in MDSCs and they utilise the L-arginine to produce urea and NO respectively [2, 77-79]. While many investigators reported that ARG1 and iNOS production was associated with an inhibition of the proliferation of T lymphocytes, Bogdan described three key mechanisms underlying the iNOS-dependent immune regulation which included the modulation of signalling processes by NO, the depletion of arginine, and the alteration of accessory cell functions [71]. High levels of ARG1 expression by MDSCs can also accelerate the depletion of L-arginine in the tumour microenvironment which inhibits T cell proliferation by causing low expression of the T cell receptors (TCR) and therefore suppresses the cell-cycle in T cells [80]. However, in a more recent study, Srivastava and colleagues have shown that MDSCs inhibit T cells by uptaking cystine and depleting the microenvironment from cysteine which is an essential amino acid for T-cell activation [76]. MDSCs compete with other APCs and import cystine intracellularly and do not export the reduced product cysteine and this renders the extracellular environment low of cysteine [76]. These findings indicate that this metabolic mechanism of T cell suppression is not unique to L-arginine and that further research is required to identify new enzymes and/or products within the tumour microenvironment that the MDSCs utilise to exert their negative immune regulatory effect [2]. Blockage of this metabolic mechanism of MDSCs can serve as a new therapeutic target for tackling T cells inhibition in cancers.

Recently, it was found that NO significantly inhibits E-selectin expression on human endothelial cells and that squamous cell carcinoma (SCC) is infiltrated by iNOS⁺CD11b⁺CD33⁺CD11c⁻HLA-DR⁻ MDSCs [81]. When SSC
was treated with iNOS inhibitor N(ω)-nitro-L-arginine(L-NNA) in vitro, E-selectin expression was induced in comparable levels to imiquimod-treated SCCs [81]. These findings indicate that MDSCs are critical producers of NO and that the blockage of NO production potentially enhances T lymphocyte activation and/or recruitment by restoring vascular E-selectin expression [2].

While ARG1 and iNOS are critical players in the suppressive activity of the MDSCs, especially in the monocytic subset, ROS production has become evident as a major regulator of the suppressive activity of the granulocytic MDSCs in both mice models and human cancers [2, 40, 82-84]. In three different studies, inhibition of ROS production in vitro was associated with complete elimination of the suppressive activities of the MDSCs isolated from mice and human cancers [37, 47, 82]. In addition, the combination of NO and ROS was associated with the production of peroxynitrite that causes dysfunction of several proteins in target cells and nitration of the TCR which leads to suppression of CD8+ T cell responses [17, 85]. Recently, ROS was shown to induce both ERBB2 and ERBB3 receptors (members of epidermal growth factor receptor (EGFR) family) in vitro and in vivo via down regulation of miR-199a and miR-125b expression in ovarian cancer [86]. Although authors did not investigate the source of ROS production and only referred to it as “endogenous ROS”, these findings suggest that ROS production is associated with tumour growth and pathogenesis [2]. However, this study has provided vital details on the mechanism by which ROS induced tumour growth. It is important to investigate whether the down regulation of miR-199a and miR-125b expression is associated with MDSC accumulation in other human cancers [2].
1.6.2 COX-2 and PGE2

Cyclooxygenase-2 (Cox-2) is a key regulator factor for inflammatory response cascade. It modulates the production of prostaglandin E2 (PGE2), which is crucial for the expression of ARG-1. Mounting evidence has demonstrated that blockade of COX-2 in many mice models, leads to significant inhibition of MDSCs accumulation, and remarkable increase in immune response induced by tumour vaccine [63, 87, 88]. In addition, COX-2 blockade was shown to provide a protective role in sepsis and trauma in murine model [89-91] and modulates cytokine secretion, and CD4:CD8 ratio [92]. In a recent study, blockade of COX-2 was found to inhibit accumulation and function of MDSCs and restores T cell response in traumatic mice [93]. The implication of COX-2 over expression is well established in human lung, colon, breast and prostate cancers pathogenesis and it is produced by tumour related MDSCs [51].

Prostaglandin E2 (PGE2) is a potent mediator produced in many inflammatory conditions and by many tumour cells. While mice MDSCs express all four PGE receptors, PGE2 was the main receptor found in human MDSCs [63, 87]. PGE2 leads to upregulation of ARG1 and accordingly regulate MDSC related T cell immune suppression [2, 63, 87, 94]. Obermajer and colleagues found that PGE2 also promotes MDSC recruitment to the tumour microenvironment through the induction of stromal cell–derived factor -1 (CXCL12/SDF-1) chemokine and the induction and stabilisation of its related receptors CXCL12 (CXC chemokine ligand 12) and CXCR4 on the surface of tumour-associated MDSCs [95]. The positive feedback loop between PGE2 and COX-2 for MDSC-related immune suppression provides
an ideal target for cancer immunotherapy. Veltman et al demonstrated that COX-2 inhibition with dietary celecoxib treatment improved immunotherapy and prevented the local and systemic expansion of all MDSC subtypes in mesothelioma murine model [96]. Therefore, simultaneous blockage of the PGE2 and COX-2 loop alone or with L-arginine metabolic products provides a potential target for countering MDSCs-related T cells suppression [2].

1.6.3 TGF-β

Transforming growth factor (TGF)-β is an immunosuppressive cytokine that has been firmly associated with MDSC function and the regulation of tumour induction and expansion [97]. In a recent study for characterisation of MDSC subsets in patients with squamous cell carcinoma of the head and neck (SCCHN), it was found that CD14+HLA-DR− MDSC subsets were the highest in numbers and produced higher levels of TGF-β than other subsets [98]. Following the addition of anti-TGF-β mAb in combination with other antibodies (anti-CD86 mAb, anti-PD-L1 mAb), T-cell proliferation and IFN-γ production were partially restored [98]. This evidence indicates that MDSC is likely to be a main source for TGF-β production and that its immunosuppressive effect is mediated by various molecules including TGF-β [2]. In two other studies it was found that TGF-β production promoted tumour cell invasion and metastasis [99, 100]. In breast cancer cells, Yang et al found that after the deletion of TGF-β receptor gene type II, MDSCs infiltrated into the invasive front of tumour tissues and synergistically produced further large quantities of TGF-β1 leading to the promotion of tumour invasion and metastasis [73]. In another supporting study, depletion of Gr-1+CD11b+ MDSC subset neutralised the antitumor effect of TGF-β
This finding indicates that efficacy of TGF-β neutralisation is dependent on the presence of Gr-1+CD11b+ cells, which could serve as potential biomarker for TGF-β immune therapy [101].

1.6.4 T regulatory cells (Tregs)

Although there remains a huge debate about whether Treg cells relate to the kinetics of expansion of MDSCs, the emerging evidence indicates that MDSCs are involved in Treg cell differentiation through the production of several cytokines and/or through direct cell-cell interactions [2]. In this regard, Huang et al found that Treg induction by MDSCs was dependent on the presence of IFNγ, IL-10 cytokines and antigen-associated activation of tumour-specific T cells but was independent of the NO mechanism in tumour bearing mice [75]. These data indicate that the MDSCs can evade the immune surveillance by directly inhibiting T cell responses and inducing anergic and suppressive Tregs. Recently, tumour-infiltrating MDSCs in mice model were found to express high levels of chemokines comprising the CCR5 ligands CCL3, CCL4, and CCL5 [2]. When CCL4 and CCL5 were injected into the tumour tissue there was a remarkable increase in tumour-infiltrating Tregs, and ablation of the CCR5 caused profound decrease in Tregs [102]. CCL5 was found to be preferentially expressed on CD4+Foxp3+ Tregs in human pancreatic cancer [103], and collectively this evidence demonstrates that MDSCs recruit higher numbers of Tregs into the tumour microenvironment by secreting several chemokines [2]. In another mouse model of lymphoma, Tregs expansion was shown to be induced by MDSCs through ARG-1 and presentation of tumour-associated antigen, but not TGF-β, dependent mechanisms [104]. In contrary, granulocytic MDSC
from tumour bearing mice impaired TGF-β1-mediated generation of CD4+CD25+FoxP3+ induced Tregs (iTregs) and impeded the proliferation of natural Tregs (nTregs) without affecting FoxP3 expression [105]. This is the first evidence to, unexpectedly, show that tumour induced granulocytic MDSC impaired the generation of CD4+CD25+FoxP3+ iTregs. Therefore, MDSC may have a dual immunomodulation effect on Treg induction in cancer [2]. In addition, there is a discrepancy in evidence supporting the cell-cell interaction between MDSCs and Treg cells. One research group found that in a mouse model of lymphoma, MDSCs induced Treg expansion through ARG1 pathway and presentation of tumour associated antigens [104]. To the contrary, others reported that the number of Tregs was high throughout tumour growth and did not correlate with MDSC-related expansion, which indicated that the induction of Treg cells was not MDSC-mediated [38]. MDSCs were found to mediate the induction of Th17 response in an independent manner of MDSCs-T cell contact but via MDSC-dependent cytokines secretion [2, 106].

1.6.5 Tumour Associated Macrophages (TAM)

Tumour associated macrophages (TAMs) are a population of myeloid cells that express negative regulation of anti-tumour immune responses. Several studies from mouse and human cancer models have established a strong correlation, between the accumulation of macrophages in tumour tissues, and poor prognosis; suggesting that TAMs can be considered as key prognostic factor of cancer progression [107-109]. While the origin of macrophages at the tumour site is not fully understood; the recruitment of monocytes to the tumour micro-environment is thought to contribute to the
accumulation of TAMs [110]. The role of macrophages in an immune response is dependent on the cytokine signalling interactions and, therefore, they either effectively destroy pathogens and activate the adaptive immune system, or attenuate the inflammatory response, which mediates wound repair and tissue remodelling. Thus, these two opposing polarisation states are classified as M1 or M2 polarisation. M1 phenotype produces IL-12, IL-6, TNF-α cytokines, whereas the M2 macrophage is characterised by the production of IL-10 and TGF- β cytokines. Another distinguishable feature of polarised macrophages is the metabolism of L-arginine, where the activation of NOS2 and ARG1 are specific markers of M1 and M2 macrophages respectively [110]. Evidence has shown that TAMs share a number of characteristics with M2 macrophages including expression of immunosuppressive factors (IL-10, TGF-β, ARG1), angiogenic factors (VEGF, CCL2, FGF2, CXCL8, CXCL1, and CXCL2) and high levels of the mannose receptor, a hallmark of M2 polarisation [107, 111, 112]. In addition, TAMs are characterised by weak production of both NO and ROS and are poor antigen presenting cells. Moreover, TAMs secret several pro-tumour factors and have been observed to recruit Tregs into tumour site through secretion of CCL22 [113]. While the relationship between TAM and MDSC is not fully understood, accumulating evidence suggests that MDSCs might, partly differentiate or be related to TAMs, which can induce T cells death and hence immunosuppression [107-109]. Recruitment of TAMs into tumour tissues appears to be mediated by the signals of tumour-derived cytokines including VEGF, M-CSF and IL-10 [114, 115]. The relationship between MDSC and TAMs was demonstrated in a recent study where CD11b+Gr-1+
MDSCs from the spleens of tumour-bearing mice, migrated to the tumour site and become TAMs, that expressed high levels of ARG1 and STAT1 phosphorylation [114]. While both MDSCs and TAMs can contribute to non-specific CD8+ T cell functional suppression at the tumour tissue, TAMs up-regulate the expression of either ARG1 or iNOS depending on the tumour microenvironment [107].

1.7 The impact of MDSCs in Cancer

The impact of MDSCs on cancer is better described as a two staged effect; the first is an abnormal myelopoiesis and recruitment of MDSCs into the tumour tissue and the second is an active MDSC cytokine production and cell-cell interactions within the environment and further progression of cancer [2, 32]. Although it is not clear whether these early recruited MDSCs have the immunosuppressive features as tumour-infiltrating MDSCs, evidence from limited number of studies has shown that these myeloid cells share both the phenotype and suppressive characteristics of MDSCs [116-118]. It is worth mentioning that this stage of MDSC recruitment is tightly regulated by several soluble molecules and up regulation of STAT3 and other signalling pathways. MDSCs were found to enhance the proliferation of tumour-initiating cells, and protect pre-cancerous intestinal epithelial cells from apoptosis, in early stages of colorectal cancer [2, 119]. Similarly, the RET/PTC3 (RP3) fusion protein, an oncogene expressed in human thyroid carcinoma, was reported to induce the production of CCL2 and GM-CSF, which promoted the recruitment of CD11b+GR1+ MDSC and hence, tumour progression [120, 121]. In a most recent update on this subject, the TNF
signalling pathway was found to drive MDSC accumulation into the tumour tissue to evade the immune system in a tumour-bearing mice model [2, 122].

It has been shown that MDSCs promote tumour immune evasion by limiting T cell responses and infiltration into the tumour microenvironment [123]. However, what remains unresolved is whether MDSCs mediate antigen-specific or nonspecific suppression of T-cell responses in tumour environment [2]. While several in vitro studies demonstrated that MDSC-mediated suppression of T cells is antigen nonspecific in nature [124, 125], there is no supporting evidence from in vivo studies [2]. MDSCs can take up soluble antigens such as tumour associated antigens and present them to T cells in order to mediate an antigen-specific suppression [17, 38]. In another supporting study, the block of MHC class I molecules on the surface of MDSCs completely abrogated their inhibitory effects on T cells in vitro [126]. CD8⁺T cells suppression is also mediated strictly by the MHC class I as shown in an in vivo cancer model [127]. Corzo and colleagues reported that MDSCs express MHC class I and lack MHC class II molecules; which supports the theory that MDSCs inhibit T-cell responses in an MHC class I-restricted manner [85]. These data also indicated that the MDSC-mediated suppression at the tumour site is quite different to that which occurs in the peripheral lymphoid tissues [2]. Isolated MDSCs from tumour tissue promoted tumour growth in vitro more remarkably than respective MDSCs derived from spleen when injected into control mice [116, 128]. Although both categories shared the same morphology and phenotype, tumour MDSCs expressed high levels of NO and ARG1 and suppressed both antigen-specific and nonspecific T cells in comparison to splenic MDSCs that
can only mediate a nonspecific T cell suppression through a ROS-mediated pathway [2, 116, 128]. It remains unclear what mechanisms regulate and affect the functions of MDSCs in tumour and peripheral organs. Further investigations of factors within the tumour tissue that can influence the functions of MDSCs will open new horizons in cancer immunotherapy.

In addition to early recruitment into the tumour site and promotion of immune escape, MDSCs have been shown to play a vital role in tumour angiogenesis and metastasis. MDSCs promote cancer cell dissemination by inducing factors that lead to a pro-angiogenic manner within the tumour microenvironment [2]. In a murine model of melanoma, investigators found that MDSCs promoted cancer cell dissemination by inducing epithelial-mesenchymal transition (EMT) and CXCL5 was the main chemokine attracting the MDSCs to the tumour site [129]. In a recent murine model of CRC, abdominal surgery trauma induced high release of high-mobility group box 1 (HMGB1), in the peritoneal cavity of mice which recruited numerous MDSCs. When HMGB1 was blocked by intra-peritoneal administration with HMGB1Box-A and gemcitabine this reduced the accumulation of MDSCs in the peritoneal cavity after the operation and eliminated the peritoneal metastasis burden of colon cancer [130]. MDSCs have also been associated with cancer angiogenesis by producing matrix metalloproteinases (MMP) and enhancing VEGF bioavailability [131]. Studies have shown that MMP9 exerts an angiogenic effect during tumourigenesis by releasing VEGF from the matrix [132]. Increased tumour angiogenesis, vascular maturation, decreased tumour cell apoptosis and tumour necrosis were observed in tumour bearing mice co-injected with Gr+CD11b+ MDSCs, which produced
high levels of MMP9 and were up-regulated through VEGF secretion[131]. In vivo induction of TIMP-2 (MMP inhibitor) in human lung adenocarcinoma A-549 led to a remarkable decrease of MDSC infiltration into tumours in addition to the suppression of angiogenesis and tumour growth [133]. In contrast, increased angiogenesis, tumour growth rate and levels of tumour Gr-1^CD11b^ MDSCs and enhanced expression of VEGF in tumour-bearing TIMP-2^−/− mice were observed [133].

1.8 Potential strategies to target MDSCs in human cancers

In recent years, there has been a remarkable increase in the wealth of information regarding tumour-associated immunosuppression and the mechanisms by which immune evasion is established. It is well recognised nowadays that MDSC-mediated immunosuppression not only contributes greatly to tumour progression but is also one of the major obstacles that limits the effectiveness of cancer treatment [2]. There are therapeutic strategies that can target MDSCs and are currently being explored as shown in Figure 1. While many of the therapeutic targets have shown promising results in mice bearing tumours, the need for such effective agents in human cancers is immense and only few have been tested in the clinical setting that will be discussed.

1.8.1 Promoting MDSC differentiation

Promoting MDSCs differentiation into mature cells has proven to be an effective strategy to eliminate their immunosuppressive activities. All-trans retinoic acid (ATRA) in therapeutic levels has shown to induce MDSC differentiation into dendritic cells (DC) and macrophages and subsequently
decreased the number of MDSCs in cancer patients and in mice [2, 46, 48, 134]. Nefedova et al suggested that an upregulation of glutathione synthesis and a reduction in ROS levels were the main mechanisms of ATRA-mediated MDSC differentiation [135]. In a recent randomised clinical trial of patients with extensive stage small cell lung cancer (n=41), systemic depletion of MDSC using ATRA in combination with cancer vaccination showed a statistically significant improvement in immune response to vaccination (41.7 %, P=0.012) in comparison to the control group (zero) and the vaccination only group (20%, P=0.02) [136]. The early data from this trial strongly suggest that therapeutic depletion of MDSC can be used clinically to enhance the effect of cancer immunotherapy [2]. In another clinical study, ATRA administration in patients with metastatic RCC dramatically reduced the number of MDSCs (Lin\(^{-}\)HLA-DR\(^{+}\)CD33\(^{+}\)), increased DC to MDSC ratio in the peripheral blood and improved T-cell immune response [18]. ATRA was administered in an escalating pattern with doses of 50, 100, and 150mg/m2/d divided in 3 daily doses for 7 days and the effect of ATRA was only observed when it reached the therapeutic target >150 ng/mL [18]. In another study of patients with advanced RCC stage III/IV, ATRA therapy was associated with reversed immune suppressive effect of MDSCs and improved T cell function by promoting MDSC differentiation into antigen presenting dendritic cells (APDCs) [137]. Nonetheless, promoting MDSC differentiation is a promising approach for targeting the MDSCs and abrogating their suppressive ability [2]. Recently, MDSCs were investigated as vectors for tumour-specific oncolytic viral therapy (vesicular stomatitis virus VSV) to treat hepatic colorectal metastatic cancer in a mouse model [138]. Since MDSCs were
superior to other immune cell types in migration to tumours, VSV delivery to the tumour tissue was potentiated by binding to MDSCs and was associated with longer-term survival of tumour bearing mice, compared to systemic administration of wild-type VSV alone. Interestingly, tumour cell death was further extended by promoting MDSC differentiation into a mature phenotype [138]. Therefore, promoting MDSC differentiation is not only a promising approach for targeting the MDSCs and abrogating their suppressive ability, but can also be used as a vehicle to deliver a more targeted oncolytic viral therapy [2].

1.8.2 Inhibition of MDSC expansion

Neutralising the effect of tumour-derived factors in order to inhibit MDSC expansion has also been a target for immunotherapy. It is proposed that pharmacological elimination of VEGF receptor-positive MDSCs (CD11b*VEGFR1*) may have a significant impact on the therapeutic efficacy of cancer vaccines [2]. A significant decrease in the total number of CD11b*VEGFR1* MDSCs was observed in metastatic RCC patients treated with VEGF-specific blocking antibody (avastin) [139]. Recently, therapeutic strategy based on IL-4 receptor α (IL4Rα) signalling blockage was designed to deplete tumour-associated MDSCs and tumour-associated macrophage (TAM) in mice. However, RNA aptamer that blocks the murine IL4Rα has also been generated for human cancer models and can serve as an important cellular mechanism of inhibiting MDSCs and TAM associated tumoral immune escape in cancers [2, 140].
1.8.3 Inhibition of MDSC function

Inhibition of MDSCs function by inhibiting the signalling pathways that regulate the production of their suppressive factors is another promising target in human cancer models. COX2, which is required for the production of prostaglandin E2, induces the upregulation of ARG1 expression by MDSCs, which is essential for their suppressive function. Therefore, COX2 inhibitors were associated with down-regulation of ARG1 expression by MDSCs, which enhanced antitumour T-cell responses and the therapeutic efficacy of immunotherapy [72, 141]. Sildenafil, phosphodiesterase-5 inhibitors, was able to decrease the production of both ARG1 and iNOS of tumour-associated MDSCs and restored in vitro T cell proliferation in multiple myeloma and head and neck cancer patients [142]. However, it is not clear whether this favourable effect is likely to be observed clinically in cancer patients. ROS inhibitors have also been shown to be effective for decreasing MDSC-mediated immune suppression in murine tumour models [2]. Nitroaspirin inhibits the production of ROS, ARG1 and iNOS, thus eliminates the MDSC suppressive functions [143]. Cimetidine (histamine type-2 receptor antagonist) has recently shown to suppress lung tumour growth in a mouse model by inducing Fas and FasL expression in MDSC surface, and regulating caspase-dependent apoptosis pathway [144]. This was associated with reduced CD11b+Gr-1+ MDSC accumulation in vivo and reversed MDSC-mediated T-cell suppression, and improved IFN-γ production in vitro [144]. These novel findings can serve as a new therapeutic target for cancer immunotherapy by MDSC apoptosis modulation [2].
1.8.4 Elimination of MDSC

The effect of chemotherapy drugs on MDSCs has been only investigated in three clinical studies in human cancers. In 17 early stage breast cancer patients receiving doxorubicin–cyclophosphamide chemotherapy every 14 days, MDSCs (Lin−HLA-DR−CD33+CD11b+) were found to be significantly reduced in numbers in the peripheral blood due to the cytotoxic elimination [49]. Similarly, in a cohort study of patients with melanoma (n=77, I-IV), the taxane-based chemotherapy administration was shown to reduce the number of circulating MDSCs (HLA-DR−CD14+) [145]. Gabitass et al have also reported a significant decrease in circulating MDSCs in patients with pancreatic (n=16) and oesophagogastric (n=23) cancer post treatment with gemcitabine-based Chemotherapy (P<0.0001) [146]. Of clinical interest, the decrease in MDSC numbers was also observed in patients with advanced stages. Nonetheless, the cytotoxic elimination of MDSCs is not target specific and can affect other immune cells. Although, The role of MDSC in limiting the antitumour efficacy of the chemotherapeutic agents Gem and 5-fluorouracil (5FU) has been highlighted in a recent in vitro study [147]. The authors have shown that that 5FU and Gem induce direct activation of the pyrin domain containing-3 protein (NLRP3)-dependent caspase-1 activation complex (termed the inflammasome) in MDSC which leads to secretion of IL-1β, production of IL-17 and therefore promotes tumour growth. MDSC-derived IL-1β production could therefore blunt the beneficial effect of antitumour therapy [2]. In addition, Gem and 5FU applied higher antitumour effects in NLRP3- mice or wild-type mice treated with interleukin-1 receptor
antagonist (IL-1Ra) [147]. The in vivo relevance to these findings is to combine 5FU treatment with IL-1 inhibition.

1.10 Hypothesis

The expansion and accumulation of MDSCs constitute one of the important mechanisms of tumour immune evasion in cancer. MDSC negatively regulate the immune responses during tumour progression, inflammation, and infection. These cells circulate in the periphery and accumulate in tumour tissues. Patients with pancreatic cancer have increased levels of systemic and local MDSC in comparison with patients having benign pancreatic disease and healthy donors.

1.11 Aim of the proposed studies

The aim of this study is to characterise the phenotype of circulating and tumour-infiltrating MDSCs and set a comparative analysis across pancreatic cancer and chronic pancreatitis patients and healthy individuals. The other aims are to evaluate the functional status of the MDSC subpopulations using arginase 1 activity, and the clinical significance of MDSCs in pancreatic disease progression.
Table 1: The phenotype and functional characteristics of MDSCs in mice

<table>
<thead>
<tr>
<th></th>
<th>Monocytic</th>
<th>Granulocytic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice</strong></td>
<td>CD11b+Ly6G&lt;sub&gt;low&lt;/sub&gt;-Ly6C&lt;sub&gt;High&lt;/sub&gt; [38] [39] [40]</td>
<td>CD11b+Ly6G&lt;sub&gt;high&lt;/sub&gt;Ly6C&lt;sub&gt;low&lt;/sub&gt; [34] [38, 40]</td>
</tr>
<tr>
<td><strong>Features</strong></td>
<td>Differentiate into mature DC and macrophages [34]</td>
<td>Do not differentiate into DC and macrophages [34]</td>
</tr>
<tr>
<td></td>
<td>More immunosuppressive than granulocytic on a per cell basis [38] [39, 148]</td>
<td>Less immunosuppressive than monocytic cells [38] [39, 148]</td>
</tr>
<tr>
<td></td>
<td>Less expansion in cancer [149]</td>
<td>Greater expansion in cancer [149]</td>
</tr>
<tr>
<td></td>
<td>Account for &lt;25% of all MDSC [149]</td>
<td>Account for 75% of all MDSC [149]</td>
</tr>
<tr>
<td></td>
<td>Suppress antigen-specific CD8&lt;sup&gt;+&lt;/sup&gt;T cell proliferation via: [34] [38]</td>
<td>Suppress antigen-specific CD8&lt;sup&gt;+&lt;/sup&gt;T cell proliferation via: [34] [38]</td>
</tr>
<tr>
<td></td>
<td>1- iNOS- mediated mechanism</td>
<td>1- ROS-mediated mechanism</td>
</tr>
<tr>
<td></td>
<td>2- Partially via IFN-γ/STAT1-dependant</td>
<td>2- Or IFN-γ/STAT1-dependant</td>
</tr>
</tbody>
</table>

iNOS: inducible nitric oxide synthase; IFN-γ: Interferon gamma; STAT: signal transducer and activator of transcription.
### Table 2: Phenotype of MDSC subsets in human cancers

<table>
<thead>
<tr>
<th>MDSC subset</th>
<th>Phenotype</th>
<th>Cancer Type</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclassified</td>
<td>LIN–HLA-DR– CD33+CD11b+</td>
<td>Breast</td>
<td>Peripheral blood</td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>CD14+/Arginase+</td>
<td>Lung</td>
<td></td>
<td>[150]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal</td>
<td></td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pancreatic</td>
<td></td>
<td>[151]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Esophageal</td>
<td></td>
<td>[49, 151]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric</td>
<td></td>
<td>[49, 151]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple myeloma</td>
<td></td>
<td>[142]</td>
</tr>
<tr>
<td>CD11b+CD14- CD15-</td>
<td>Melanoma</td>
<td>Peripheral blood and tumour tissue</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>----------------------------------</td>
<td>------</td>
<td></td>
</tr>
</tbody>
</table>

**Granulocytic**

<table>
<thead>
<tr>
<th>C11b+CD14-CD15+CD66b+VEGFR1+</th>
<th>Renal</th>
<th>Peripheral blood</th>
<th>[52]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15++ IL-4R+CD124</td>
<td>Colon</td>
<td>Peripheral blood</td>
<td>[152]</td>
</tr>
<tr>
<td>CD11b+CD14 -CD15+CD33+</td>
<td>Lung</td>
<td>Peripheral blood</td>
<td>[153]</td>
</tr>
<tr>
<td>CD11b+CD14-CD15^int</td>
<td>Melanoma</td>
<td>Peripheral blood and tumour tissue</td>
<td>[53]</td>
</tr>
</tbody>
</table>

**Monocytic**

<table>
<thead>
<tr>
<th>CD14+CD11b+HLA-DR&lt;sub&gt;low&lt;/sub&gt;</th>
<th>Melanoma</th>
<th>Peripheral blood</th>
<th>[152]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td></td>
<td></td>
<td>[154]</td>
</tr>
<tr>
<td>HCC</td>
<td></td>
<td></td>
<td>[155]</td>
</tr>
<tr>
<td>Head &amp; Neck</td>
<td></td>
<td></td>
<td>[142]</td>
</tr>
<tr>
<td>CD14+ IL-4R+CD124</td>
<td>Colon</td>
<td>Peripheral blood</td>
<td>[152]</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
<td>------------------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td></td>
<td>[152]</td>
</tr>
<tr>
<td>CD14+</td>
<td>Melanoma</td>
<td>Peripheral blood and tumour tissue</td>
<td>[53]</td>
</tr>
</tbody>
</table>

MDSC: Myeloid-derived suppressor cell; VEGFR1: vascular endothelial growth factor receptor 1; int: intermediate; HCC: Hepatocellular carcinoma.
Table 3: Factors implicated in the expansion and/or activation of MDSCs in cancer

<table>
<thead>
<tr>
<th>MDSC Expansion/activation Process</th>
<th>Factors</th>
<th>Production of factors</th>
<th>Signalling pathways</th>
<th>Cellular effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expansion</td>
<td>COX2 [156]</td>
<td>Tumour cells</td>
<td>JAK protein and STAT3</td>
<td>Myelopoiesis stimulation</td>
</tr>
<tr>
<td></td>
<td>Prostaglandins [87, 156]</td>
<td></td>
<td></td>
<td>Inhibiting the differentiation of MMC</td>
</tr>
<tr>
<td></td>
<td>SCF [157]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-CSF [134, 158]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-6 [159]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GM-CSF [160]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VEGF [161]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation</td>
<td>IFNγ [66]</td>
<td>Activated T cells and tumour stromal cells</td>
<td>STAT6, NF-κB</td>
<td>MDSC activation</td>
</tr>
<tr>
<td></td>
<td>IL-13 [40]</td>
<td></td>
<td>STAT1</td>
<td>Upregulation of</td>
</tr>
<tr>
<td></td>
<td>IL-4 [67]</td>
<td></td>
<td></td>
<td>iNOS and arginase</td>
</tr>
<tr>
<td></td>
<td>TGF-β [68, 73]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Potential therapeutic strategies for targeting MDSCs. There are four potential therapeutic strategies to eliminate immunosuppressive activity of MDSCs. 1. Promoting MDSCs (monocytic subset) differentiation into mature dendritic cells (DC) and macrophages. 2. Inhibition of MDSC expansion by neutralising the effect of tumour-derived activating and expanding factors such as: COX2, PGE2, SCF, IL-6, GM-CSF, VEGF, TGF-β, IFNγ etc. 3. Inhibition of MDSC function by inhibiting the signalling pathways that regulate the production of their suppressive factors which leads to decrease the production of arginase1 (ARG1), inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS). 4. Pharmacological elimination of MDSC by targeting cell’s DNA and cell membrane leading to cell apoptosis.
Chapter 2
Chapter 2: Methodology

2.1 Study design

This was a prospective study characterising phenotype of MDSC subsets and their functional state in patients with PADC in comparison to patient with CP and healthy controls. Blood and tumour tissue samples were collected at North Manchester General Hospital (NMGH) by Dr Yazan Khaled, from participants following formal consent to participate in this study from April 2012 to March 2013. An ethical approval for this study was obtained from all laboratory investigations were performed at the University of Salford (UOS).

2.2 Ethical considerations

- REC approval: This study was approved by the relevant research ethics committees (RECs), Appendix 1. These were Northwest Centre for Research Ethics Committee (NREC), the University of Manchester (UOM) and UOS.

- Research and Development (R&D) approval: After the REC approval was received from three sites, the study proposal was submitted to the relevant R&D of NMGH whom also approved (Appendix 2).

The principles of The International Conference on Harmonization-Guidelines for Good Clinical Practice (ICH-GCP guidelines 1996) were applied to this study.

2.3 Consent

The studies were explained to patients at least 24hrs before participation in the study, and the option not to participate was always offered. An informed
written consent was always obtained. The patient information sheet for each group is displayed in Appendix (3-4) and the consent form in Appendix 5. A copy of the signed consent form was retained in the patients’ clinical case notes, another was given to the patients and a third was held in the research study notes by the researcher. All data recorded during the study were treated in confidence.

2.4 Data collection and protection

Data Protection Act (DPA) and NHS code of confidentiality and the National Research Ethics Service’s Guidance for Applicants were followed. The personal data of all participants were recorded at time of recruitment into the study in a separate password protected digital file and each participant was assigned a study code number that was used in the data files, in the CRF and to identify samples. All laptops used in the study were encrypted. Hard copies of the patients’ information were stored in fire resistant lockable filing cabinets on NHS premises and these will only be accessible to the chief investigator and the research fellow. All samples collected from patients were anonymised and coded so that no patients can be identified; no personal data would be needed when transferring and analysing data. All the samples and the data collection were performed by the members of the research team. The researchers collected data accurately, efficiently and according to the agreed design of the research project, and ensured that data were stored in a secure and accessible form.
2.5 Study populations

There were three groups of patients:

Group 1: The control group of healthy subjects

Group 2: Patients with chronic pancreatitis

Group 3: Patients with pancreatic cancer

The Consultant HepatoPancreatoBiliary (HPB) Surgeon and the research fellow identified patients with confirmed or suspected pancreatic cancer. These patients were discussed at weekly HPB multidisciplinary team (MDT) meetings at North Manchester General Hospital (NMGH), and this acted as a suitable medium for identifying suitable patients. Control patients with chronic pancreatitis or benign pancreatic neoplasms that were due to undergo pancreatic surgery were also identified through the benign MDT monthly meetings at NMGH, while control subjects undergoing laparoscopic cholecystectomy or hernia repair were identified from within the HPB practice at NMGH. The control group consisted of healthy patients who underwent elective surgery for benign conditions such as laparoscopic cholecystectomy or hernia repair.

2.6 Inclusion criteria

- Consent ing adults aged ≥18 years

- Non-septic patients; i.e. absence of pyrexia, leucocytosis, and abdominal tenderness
- Patients with pancreatic cancer including those with operable disease undergoing resection and non-operable undergoing palliative therapy
- Patients with chronic pancreatitis including those with operable disease undergoing resection and non-operable undergoing conservative management
- Histological confirmation of pancreatic adenocarcinoma post operatively
- Histological confirmation of chronic pancreatitis post operatively

2.7 Exclusion criteria

- Septic patients, as sepsis may affect the levels of MDSC
- Patients receiving chemotherapy, radiotherapy or supra-physiological doses of steroids or other immunosuppressive therapy as these may affect the levels and functional state of MDSC
- Pregnant patients
- Patients refusing consent

2.8 Blood and tissue samples

Blood samples were collected in a 50ml Falcon™ tube (BD biosciences, UK) containing a final concentration of 1000IU/ml heparin. The samples were transported immediately to the laboratory to be processed on the same day. Two hundred microliter (μl) of whole blood was taken for MDSC analysis as explained below. Blood isolation was performed to obtain plasma and peripheral blood mononuclear cells (PBMCs) for other investigations.
Tumour tissues were collected in two sterile tubes; one tissue piece was placed in a tube supplemented with Roswell Park Memorial Institute RPMI-1640 (Sigma, Dorset, UK) and kept on ice until arrival at the laboratory to be processed immediately. The other tube containing tumour tissue was fixed in formalin to be processed after 24 hours for formalin fixed paraffin embedded section [162].

2.8.1 Whole blood staining and cell isolation

Two hundred microliter (μl) of whole blood was taken for MDSC analysis divided into 100 μl for non-stained tube and 100 μl for stained tube with mouse anti-human monoclonal antibodies (mAb) for different MDSC markers. Sample tubes were vortexed well and incubated for 25 minutes at 4°C. After the incubation, lysis buffer (BD FACS lysing solution) was added to lyse the red blood cells (RBCs) and then incubated for 15 minutes at room temperature. The samples were washed twice with phosphate buffered saline (PBS). The pellet was resuspended in 300μl of flow cytometry buffer (FCB) before analysis. Blood samples were processed to isolate peripheral blood mononuclear cells (PBMCs) for other investigations. Analysing MDSCs in fresh whole blood is currently recommended (to include granulocytic MDSCs) and no dead cells/debris will be included in the analysis. Many flow cytometric methods don’t use viability dye especially if the analysed samples are fresh (like using fresh whole blood) and dead cells/debris can be excluded based on forward and side scatter characteristics [162].
2.8.2 Enzymatic disaggregation of tissue samples

Tumour samples were processed immediately in a microbiological safety class II cabinet to avoid any possible contamination. Tumour tissue was washed by RPMI-1640 or PBS (pH 7.4) in a Petri dish to remove any red blood cells. Tumour tissue was then minced in small pieces approximately 2 to 4 mm$^3$. Fragments of the tumour tissue were snap-frozen and kept in cryovials and stored in liquid nitrogen for further histological analysis. The rest of tumour tissue pieces were collected in sterile tube and resuspended in enzymes cocktail. Tissue specimens from patients with PC and CP were minced under aseptic techniques into 2-4 mm pieces, re-suspended in enzymatic cocktail and incubated on roller mixer for overnight at room temperature. Then the cell suspension was passed through a Falcon™ 100μm cell strainer (BD Biosciences, Oxford, UK) to remove any large aggregates and debris. The suspension was washed twice with RPMI-1640 and centrifuged at 300g for 5 minutes before counting and staining.

The method used to digest the tissue samples and to analyse the tumour-infiltrating MDSCs was chosen by comparing the frequency of MDSC subsets for two tumour samples with the different methods. The suspension of the minced tumour in 1mg/ml collagenase (Sigma-Aldrich, UK), 100ug/ml of hyaluranidse type V (Sigma-Aldrich, UK) and 30IU/ml of DNase type I (Sigma-Aldrich, UK), and incubation on roller mixer overnight at room temperature was chosen to study tumour-infiltrating MDSCs [162].
2.9 Reagents

To best of our knowledge, PDAC do not express oestrogen receptors and therefore RPMI-1640 phenol red (Sigma, Dorset, UK) was used as transporting media for tissue samples. Monoclonal antibodies (mAb) included mouse anti-human monoclonal antibodies: anti-Lin-FITC, anti-CD11b-APC-Cy7, anti-CD14-PerCP-Cy5.5, anti-CD15-PE-Cy7, anti-CD33-APC and anti-HLA-DR-PE. All these antibodies were purchased from BD Bioscience (BD Pharmingen). Arginase activity was determined using arginase 1 mAbs from R&D systems (Oxford, UK).

2.10 Antibodies and flowcytometric analysis

Flow cytometric analysis was performed simultaneously on fresh whole blood and tissue suspension following enzymatic disaggregation. MDSC subsets were determined using the following antibodies: anti-Lin-FITC, anti-CD11b-APC-Cy7, anti-CD14-PerCP-Cy5.5, anti-CD15-PE-Cy7, anti-CD33-APC and anti-HLA-DR-PE. All the mAbs were purchased from BD Biosciences. Six colour flow cytometric analysis was performed on a BD FACSVerse flow cytometer. Analysis of the flow cytometric data was performed using the BD FACSuite software. This state-of-art technology generates accurate cell frequencies count for the gated cell population. The frequencies were entered into a GraphPad Prism 5.0 software sheet and analysed. Data collected were double checked for accuracy before statistical analysis [162].
2.11 Functional characterisation of MDSCs

Arginase activity was determined using arginase 1 mAbs from R&D systems (Oxford, UK). Intracellular staining was performed on 100μl of fresh whole blood with Sheep anti-Human/Mouse Arginase 1 Fluorescein-conjugated polyclonal antibody. We first added antibodies for surface markers (CD11b-APC-Cy7, CD14-PerCP-Cy5.5, CD15-PE-Cy7, CD33-APC and HLA-DR-PE) to the whole blood sample then vortexed the sample tubes well and incubated for 25minutes at 4°C. Due to our flow cytometer configuration, we were only able to do six colours staining. Therefore, we did not use lineage staining because anti-Lin antibody is FITC as well and we can't use the two FITC antibodies together. RBCs were then lysed using BD FACS lysing solution. The samples were washed twice with PBS and the pellet was resuspended in 1ml of freshly prepared eBioscience Fixation/permeabilisation working solution. Tubes were then incubated for 45minutes at 4°C in the dark. The samples were washed twice with permeabilisation buffer. Arginase 1 mAb was then added to the cell suspension and incubated in the dark for 30minutes then washed twice with permeabilisation buffer. The pellet was resuspended in 300μl of FCB for analysis [162].

2.12 Statistical Analysis

Comparisons of the frequencies of the following subsets in the study groups were performed: Lin-HLA-DR-, Lin-HLA-DR-CD33+11b+, Lin-HLA-DR-CD33+11b+CD15+, Lin-HLA-DR-CD14+, HLA-DR-^low^CD14+, HLA-DR-^low^CD33+. Statistical analysis was performed on GraphPad Prism 5.0
software (GraphPad Software, United States). Unpaired Student’s t test (Mann-Whitney test) was used to assess the differences between the study groups. Non-parametric Spearman test was used to assess the correlation between circulating MDSCs and cancer clinical stage. P value ≤0.05 was considered statistically significant. The data are presented as medians (range).
Chapter 3
Chapter 3: Results

3.1 Patients analysis

The total number of patients recruited, including the control group, was 52 patients (25 males, 27 females) from April 2012 to March 2013. Peripheral blood samples were collected from PC patients ($n=24$) and CP patients ($n=12$). Sixteen age-matched healthy donors were used as controls. Tumour ($n=7$) and benign ($n=7$) pancreatic tissue samples were collected from patients who underwent surgery. A further 16 patients were considered for the study but excluded for reasons outlined in Table 4. The limited number of tissue samples from patients was due to the low number of patients with chronic pancreatitis who needed surgical intervention or patients who were deemed inoperable due to locally advanced pancreatic cancer. Acute inflammatory conditions in addition to the refusal of some patients to participate in the study were also limiting factors.

The median age has been found to have no statistically significant difference across the three studied groups: HD 51 (24-89), CP 49 (23-72) and PC 64.5 (41-85), Figure2.

The aetiology of CP was idiopathic in nine patients (75%) and alcohol-related in three patients (25%). The preoperative details, investigations and disease characteristics for the CP study group are shown in Table 5.

All patients with PC were diagnosed for the first time and had not been previously treated. The preoperative details, investigations and disease characteristics for the CP study group are shown in Table 6. The levels of
tumour biomarkers were also collected for PC patients before the first dose of chemotherapy or surgery for clinical correlation with the levels of MDSCs.

3.2 Frequency of granulocytic MDSCs is significantly elevated in the peripheral blood of patients with PC

Previous reports have described circulating MDSCs in human cancers as monocytic (HLA-DR-CD14+) [163] or granulocytic (CD14-CD15+) [164]. We thus aimed to study all the subsets of circulating MDSCs using multicolour flow cytometric analysis of whole blood from patients with PC, CP and HDs using all the previously mentioned markers. We defined granulocytic MDSC as Lin-HLA-DR-CD33+CD11b+CD15+ and monocytic MDSC as Lin-HLA-DR-CD14+. Representative Flowcytometric data of a normal HD, one patient with CP and one with PC are shown in Figures 3, 4, 5 respectively. The frequency of circulating Lin-HLA-DR-CD33+CD11b+CD15+ subset was significantly higher in PC patients compared with HDs (8.86% vs. 1.33%; \( P=0.0003 \)) but was not statistically higher in comparison with CPs (\( P=0.54 \)) as shown in Figure 5. In addition, there was no statistical difference in the levels of the Lin-HLA-DR-CD33+CD11b+CD15+ between HDs and CP patients. The frequency of Lin-HLA-DR-CD33+CD11b+CD15+ granulocytic subset was greater than monocytic Lin-HLA-DR-CD14+ in the peripheral blood of patients with PC (8.86% vs. 0.89%, \( P=0.004 \)) and in those with CP (8.95% vs. 1.35%, \( P=0.003 \)). However, no statistical difference was found in the frequency of circulating Lin-HLA-DR-CD14+ subset when comparing the three study groups. A more detailed description of the frequency of different MDSC subsets in peripheral blood of HDs, CP and PC patients is shown in
Table 7. Of note, the peripheral blood levels of monocytic MDSCs (HLA-DR-CD14+) were lower in the blood of PC (0.8%) group in comparison to HDs (1.3%) and CP (1.6%) groups, although this difference did not reach statistical significance [162].

3.3 Arginase 1 (ARG1) expression in the circulating MDSCs

High levels of ARG1 expression by MDSCs can accelerate the depletion of L-arginine in the tumour microenvironment, which subsequently inhibits T-cell proliferation by causing low expression of T-cell receptors and thus suppression of the cell cycle in T cells [165]. Others demonstrated that ARG1-producing MDSCs are granulocytic and they are increased in the circulation of human cancers[52, 166]. To investigate whether the circulating MDSCs express ARG1 in PC, flowcytometric based assay for ARG1 expression was used. HLA-DR- against side scatter (SSC) was first gated. Then ARG1 expression in CD33+, CD11b+, CD15+ and CD14+ in circulating MDSCs was determined. If circulating MDSCs in PC are predominantly granulocytic as we have shown in Figure 5, then these cells should express ARG1 but not the monocytic subsets. Figure 7 shows that ARG1 was only expressed in CD33+, CD11b+ and CD15+. This confirms that ARG1 expression is characteristic for granulocytic MDSCs in pancreatic cancer [162].

3.4 Myeloid cells infiltrating pancreatic tumour tissue are predominantly granulocytic MDSC subset

The pancreatic tumour tissue infiltrate was examined for presence of myeloid cells and was assessed for the expression of the same markers used to...
characterise circulating myeloid cells (Lin, HLA-DR, CD33, CD11b, CD14, and CD15) by flow cytometry in the tumour cell suspensions following enzymatic disaggregation (ED) of fresh surgically resected pancreatic tumour tissues of seven patients with stage I/II PC. Seven benign pancreatic tissues, surgically excised of patients who underwent surgical intervention for the treatment of CP, were analysed in similar manner after ED. The percentage of Lin-HLA-DR-CD33+CD11b+CD15+ MDSC subset in the tissue-infiltrating myeloid cells was markedly increased in the tumour tissue when compared to the matched benign tissue (11.11% vs. 0.20%, \( P=0.037 \)) (Figure 8). Representative flowcytometric plots of one patient with PC and one with CP are shown in Figure 9 and 10 respectively. Although no statistical significant difference was reached in the frequency of infiltrating monocytic Lin-HLA-DR-CD14+ subset in tumour tissues when compared to the benign samples (3.85% vs. 0.25%, \( P=0.209 \)), a 4-fold increase was observed in infiltrating Lin-HLA-DR-CD14+ in comparison to the respective circulating group (3.85% vs. 0.885, \( P=0.072 \)). However, in benign pancreatic tissue samples, there was no increase in the frequency of infiltrating monocytic Lin-HLA-DR-CD14+ when compared to their respective circulating subsets (0.25% vs. 63%, \( P=0.84 \)). The frequency of tumour-infiltrating HLA-DR-CD33+CD11b+CD15+ MDSC subset was markedly higher than the monocytic Lin-HLA-DR-CD14+ subset but this did not reach a statistical significance (11.11% vs. 3.85%, \( P=0.073 \)). Therefore, this indicates that these cells are indeed tissue/tumour-infiltrating MDSCs, and not just a representation of the blood contained within the tissue samples. Further details of the frequency of the different
tissue-infiltrating MDSC subsets that we examined are illustrated in Table 8 [162].

3.5 Circulating MDSCs do not correlate with clinical cancer stage

PC patients were divided by clinical cancer stage into an operable group (Stage I/II, n=6) and inoperable group (Stage III/IV, n=18). The percentage of circulating MDSCs in patients with operable tumours was comparable to that of patients with inoperable disease, and this was applicable to both Lin-HLA-DR-CD33+CD11b+CD15+ subset (8.5% vs. 8.95%, P=0.45) and monocytic Lin-HLA-DR-CD14+ (1.32% vs. 0.63%, P=0.105).

In addition, we also analysed the correlation between the circulating MDSCs and pancreatic tumour size and preoperative serum concentration of CA19-9 and CEA biomarkers. Of the seven cases analysed, six had the record of the tumour size after resection. No correlation was observed between the frequency of circulating MDSCs and tumour size (P=0.86) or with the serum concentration of CA19-9 and CEA cancer biomarkers (P=0.77, 0.86 respectively) [162].
Table 4

Table 4: Patients considered for the study but excluded and the reasons

<table>
<thead>
<tr>
<th>Group</th>
<th>Reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>Refused consent (n=7)</td>
</tr>
<tr>
<td>CP</td>
<td>Acute on chronic pancreatitis (n=4)</td>
</tr>
<tr>
<td>PC</td>
<td>Unable to give blood samples (n=3)</td>
</tr>
<tr>
<td></td>
<td>Recent chemotherapy (n=2)</td>
</tr>
</tbody>
</table>

HD: healthy donor; CP: chronic pancreatitis; PC: pancreatic cancer
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (range)</td>
<td>49 (23-72)*</td>
</tr>
<tr>
<td>Gender (Male:Female)</td>
<td>5:7</td>
</tr>
<tr>
<td>Etiology</td>
<td>Idiopathic, n=9</td>
</tr>
<tr>
<td></td>
<td>Alcoholic, n=3</td>
</tr>
<tr>
<td>Investigations to confirm diagnosis</td>
<td>CT and ERCP, n=12</td>
</tr>
<tr>
<td>Co-morbidities</td>
<td>Nil, n=5</td>
</tr>
<tr>
<td></td>
<td>Exocrine insufficiency, n=4</td>
</tr>
<tr>
<td></td>
<td>Endocrine &amp; exocrine insufficiency, n=2</td>
</tr>
<tr>
<td></td>
<td>DM II, n=1</td>
</tr>
<tr>
<td>Preoperative CA19-9 (0-37 U/ml)</td>
<td>54</td>
</tr>
</tbody>
</table>

CP: chronic pancreatitis; DM II: Diabetes mellitus type 2. *Data shown represent median (range).
Table 6

Table 6: Patients demographics and disease characteristics of PC

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>$n=24$</td>
<td></td>
</tr>
<tr>
<td>Age (median)</td>
<td>64.5 (41-85)*</td>
<td></td>
</tr>
<tr>
<td>Gender (Male: Female)</td>
<td>13:11</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Tumour size (cm)</td>
<td>3.2 (1.9-6.1)*</td>
<td></td>
</tr>
<tr>
<td>Preoperative CA19-9 (0-37 U/ml)</td>
<td>399 (77-1230)*</td>
<td></td>
</tr>
<tr>
<td>Preoperative CEA (&lt;2.5 ng/ml)</td>
<td>5 (5-13)*</td>
<td></td>
</tr>
<tr>
<td>Tumour site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head of pancreas</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Body of pancreas</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Tail of pancreas</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Well/moderate</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Poor/undifferentiated</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

PC: pancreatic cancer; CA19-9: cancer antigen 19-9; CEA: carcinoembryonic antigen. *Data shown represent median (range).
Table 7: Frequency of MDSC subsets in the peripheral blood of healthy donors, chronic pancreatitis and pancreatic cancer patients

<table>
<thead>
<tr>
<th></th>
<th>PC</th>
<th>CP</th>
<th>HDs</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin-HLA-DR-</td>
<td>2.7</td>
<td>1.9</td>
<td>4.3</td>
<td>A=0.14, B=0.59, C=0.79</td>
</tr>
<tr>
<td></td>
<td>(0.7-17.5)*</td>
<td>(1-90.7)*</td>
<td>(1.0-14.6)*</td>
<td></td>
</tr>
<tr>
<td>Lin-HLA-DR-CD33+11b+</td>
<td>8.1</td>
<td>11.8</td>
<td>8.7</td>
<td>A=0.6, B=0.85, C=0.89</td>
</tr>
<tr>
<td></td>
<td>(1.4-27.6)*</td>
<td>(0.5-34.2)*</td>
<td>(0-19.2)*</td>
<td></td>
</tr>
<tr>
<td>Lin-HLA-DR-CD33+11b+CD15+</td>
<td>8.8</td>
<td>8.9</td>
<td>1.4</td>
<td>A=0.0003, B=0.65, C=0.13</td>
</tr>
<tr>
<td></td>
<td>(0-91.3)*</td>
<td>(0-33.7)*</td>
<td>(0-14.5)*</td>
<td></td>
</tr>
<tr>
<td>Lin-HLA-DR-CD14+</td>
<td>0.8</td>
<td>1.6</td>
<td>1.3</td>
<td>A=0.47, B=0.43, C=0.63</td>
</tr>
<tr>
<td></td>
<td>(0.2-6.6)*</td>
<td>(0.4-6.3)*</td>
<td>(0.16-3.5)*</td>
<td></td>
</tr>
<tr>
<td>HLA-DR-/lowCD14+</td>
<td>0.9</td>
<td>2.2</td>
<td>1.8</td>
<td>A=0.41, B=0.83, C=0.79</td>
</tr>
<tr>
<td></td>
<td>(0.2-7.5)*</td>
<td>(0.2-37.1)*</td>
<td>(0.6-6.7)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>CP</td>
<td>HDs</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>HLA-DR-\textsuperscript{Low}CD33\textsuperscript{+}</td>
<td>10.5</td>
<td>14.8</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1.9-31.2)*</td>
<td>(0.4-93.1)*</td>
<td>(1.4-23.9)*</td>
<td></td>
</tr>
</tbody>
</table>

A=0.69, B=0.818, C=0.89

PC: pancreatic cancer; CP: chronic pancreatitis; HDs: healthy donor. P value: A= (PC vs. HDs); B= (PC vs. CP); C= (CP vs. HDs). *Data shown represent median (range)
Table 8: Frequency of tissue infiltrating MDSC subsets in benign and malignant pancreatic tissue

<table>
<thead>
<tr>
<th></th>
<th>PC</th>
<th>CP</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin-HLA-DR-</td>
<td>57.3</td>
<td>91.82</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(9.4-90.9) *</td>
<td>(10.6-95.8) *</td>
<td></td>
</tr>
<tr>
<td>Lin-HLA-DR-CD33+11b+</td>
<td>2.9</td>
<td>0.35</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>(1.4-27.6) *</td>
<td>(0.03-13.7) *</td>
<td></td>
</tr>
<tr>
<td>Lin-HLA-DR-CD33+11b+CD15+</td>
<td>11.1</td>
<td>0.2</td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td></td>
<td>(0.5-72.3) *</td>
<td>(0.03-13.6) *</td>
<td></td>
</tr>
<tr>
<td>Lin-HLA-DR-CD14+</td>
<td>2.5</td>
<td>0.3</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>(0.19-5.07) *</td>
<td>(0.1-17.5) *</td>
<td></td>
</tr>
<tr>
<td>HLA-DR-\text{low}CD14+</td>
<td>2.7</td>
<td>2.3</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>(0.5-5.1) *</td>
<td>(0.1-14.1) *</td>
<td></td>
</tr>
<tr>
<td>HLA-DR-\text{low}CD33+</td>
<td>6.9</td>
<td>11.5</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>(0.82-25.5) *</td>
<td>(0.31-13.6) *</td>
<td></td>
</tr>
</tbody>
</table>

PC: pancreatic cancer; CP: chronic pancreatitis; HDs: healthy donors. *Data shown represent median (range).
Figure 2

Figure 2: Age: Box plot representation of age distribution in the three studied groups and shows that there was no statistically significant difference between the patients in the CP, PC and HD study populations. Bar represents median in each group. HD: healthy donor; CP: chronic pancreatitis; PC: pancreatic cancer.
Figure 3

Figure 3: Levels of circulating MDSCs in healthy donors. Flow cytometric evaluation of Lin, CD33, CD11b, CD15 and CD14 in whole blood. An example of representative dot plots is shown in this figure. Gates were set based on negative controls. Numbers represent the percentages from the original populations gated. P (number) above each FACS plots indicate the population gated that was analysed. The axis of each FACS plot represents the marker analysed.
Figure 4

Figure 4: Levels of circulating MDSCs in chronic pancreatitis. Flow cytometric evaluation of Lin, CD33, CD11b, CD15 and CD14 in whole blood. An example of representative dot plots is shown in this figure. Gates were set based on negative controls. Numbers represent the percentages from the original populations gated. P (number) above each FACS plots indicate the population gated that was analysed. The axis of each FACS plot represents the marker analysed.
**Figure 5**

**Figure 5**: Levels of circulating MDSCs in pancreatic cancer. Flow cytometric evaluation of Lin, CD33, CD11b, CD15 and CD14 in whole blood. An example of representative dot plots is shown in this figure. Gates were set based on negative controls. Numbers represent the percentages from the original populations gated. P (number) above each FACS plots indicate the population gated that was analysed. The axis of each FACS plot represents the marker analysed.
Figure 6: Scatter plot of the percentage of Lin-HLA-DR-CD33+CD11b+CD15+ in the blood of patients with pancreatic cancer, compared to chronic pancreatitis patients and healthy donors. Bar represents median in each group. HD: healthy donor; CP: chronic pancreatitis; PC: pancreatic cancer.
Figure 7: Arginase 1 expression in different subsets of circulating MDSC in patients with PC. Flow cytometric evaluation of ARG1 expression in CD33, CD11b, CD15 and CD14 in whole blood is shown in the representative dot plots. Gates were set based on negative controls. Numbers represent the percentages from the original populations gated. P (number) above each FACS plots indicate the population gated that was analysed. The gate was first set on HLA-DR negative against side scatter as shown in the top dot plot. Next, the CD11b+ & CD33+ (right) and CD14+ & CD15+ (left) subsets were identified. The expression of ARG1 in each subset was then determined as shown in the bottom dot plots.
Figure 8: Scatter plot of the percentage of Lin-HLA-DR-CD33+CD11b+CD15+ in the tissue of benign and cancer samples. Bar represents median in each group. CP: chronic pancreatitis; PC: pancreatic cancer.
Figure 9

Figure 9: Levels of tumour-infiltrating MDSCs in patients with pancreatic cancer. Flow cytometric evaluation of Lin, CD33, CD11b, CD15 and CD14 in tissue sample. Gates were set based on controls. Numbers represent the percentages from the original populations gated. P (number) above each FACS plots indicate the population gated that was analysed. The axis of each FACS plot represents the marker analysed.
**Figure 10**: Levels of tumour-infiltrating MDSCs in patients with chronic pancreatitis. Flow cytometric evaluation of Lin, CD33, CD11b, CD15 and CD14 in tissue sample. Gates were set based on controls. Numbers represent the percentages from the original populations gated. P (number) above each FACS plots indicate the population gated that was analysed. The axis of each FACS plot represents the marker analysed.
Chapter 4
Chapter 4: Discussion

4.1 Introduction

The previous chapter has discussed the findings regarding the circulating and tissue infiltrating levels of MDSCs in pancreatic cancer patients. The original aim of the study was to characterise the phenotype of circulating and tumour-infiltrating MDSCs in pancreatic cancer, with respective controls, and to acquire a deeper understanding of their functional status and the clinical significance of MDSCs in pancreatic disease progression. This characterisation of the different MDSC subsets in PC helps to enable the identification of potential pharmacological strategies to alter the function of MDSCs in order to improve the efficacy of PC treatment.

4.2 Key findings

4.2.1 Circulating and tissue-infiltrating MDSCs and their ARG1 expression in PC

This is the first study, to date, that examined the frequency and function of different MDSC subsets in blood and tumour tissue of patients with pancreatic cancer in comparison with HDs and benign pancreatic disease in the form of CP. Circulating and tissue-infiltrating granulocytic MDSCs were significantly elevated in the PC patients when compared to respective controls.

Previous work established the presence of granulocytic MDSCs in the circulation of different human cancers including renal, lung cancer (CD11b+CD14-CD15+) [72, 167], breast, colon, and pancreatic cancers
(CD15+) [47]. On the other hand, circulating monocytic HLA-DR-CD14+ was found elevated in patients with hepatocellular carcinoma, melanoma, prostate cancer, and multiple myeloma [155, 163, 168, 169]. We found that Lin-HLA-DR-CD33+CD11b+CD15+ subset was significantly elevated in the blood of PC patients when compared to HDs. This is concordant with the findings of Gabitass et al who reported a statistically significant higher frequency of circulating MDSCs in the blood of patients with PC (n=46) compared to HDs (2.1, $P<0.001$) [151]. However, they only defined MDSC as Lin-HLA-DR-CD33+CD11b+ with no further characterisation of granulocytic or monocytic subsets. In addition, they performed their phenotypical analysis on PBMCs, which could account for the relatively lower levels in comparison to our study [162]. A unique and important finding of our study is that there was no statistical difference in the median of circulating Lin-HLA-DR-CD33+CD11b+CD15+ in the blood of PC patients when compared with CP patients. In another supporting study, Basso and colleagues demonstrated that circulating MDSCs (CD33+CD14-HLA-DR-) were significantly increased ($P=0.022$) in comparison to HDs whereas dendritic and cytotoxic T cells were reduced in PC patients [162, 170]. Other groups have described that MDSCs found in the peripheral blood of patients with renal cell carcinoma and PC, derived from PBMC, had the morphology of granulocytes [47, 72]. These MDSCs were CD11b+CD15+, expressed high levels of ARG1, and were negative for macrophage/monocytic marker CD14 [72]. In the present study, we have demonstrated that ARG1 was mainly expressed by granulocytic MDSCs in the circulating of patients with PC. Therefore, ARG1 can be used as a specific marker for the frequency of granulocytic MDSC in cancers
This finding is of significant importance in establishing specific functional MDSC markers for future planned investigations. ARG1 expression levels can be measured by quantitative reverse transcription polymerase chain reaction (QRT-PCR) instead of six colour markers that are needed for the flowcytometric analysis [162]. This is useful for large patient multicentre studies which might be a valuable tool to reduce the variability associated with day-to-day investigations [171]. Collectively, the existing evidence indicate that MDSC expansion in the circulation might favour tumour growth and progression in PC and other human cancers [170].

Our data indicate that MDSCs are expanded not only in cancer progression but also in chronic inflammatory conditions such as CP, and this supports the findings of others [162, 172]. In the last two decades, accumulating evidence has established that longstanding pre-existing CP is a strong risk factor for pancreatic cancer with an estimated rate of 5% of PC development [173]. It will be interesting to correlate the levels of MDSCs in the blood of CP patients with the disease burden and with those who develop PC. We found the levels of monocytic MDSCs (HLA-DR-CD14+) to be lower in the blood of PC group in comparison to HDs and CP groups. Although this difference did not reach statistical significance, this may indicate that monocytic MDSCs are expanded in chronic inflammatory conditions including CP. This observation was not described previously but may suggest that granulocytic and monocytic MDSCs have distinct function in humans with chronic inflammation or tumour burden. Therefore, further investigations are required to increase our understanding of the immunosuppressive characteristics of each subset in the circulation [162].
MDSCs represent a group of immature cells that are morphologically, phenotypically and functionally heterogeneous and play an important role in cancer immune evasion. This heterogeneity has created a major obstacle in generating specific MDSC markers, and this limited our understanding of their suppressive role in human cancers. However, further identification of the characteristic features of MDSC subsets in different types of human cancers dictated further investigations to redefine MDSCs according to a combination of a new set of markers, such as high levels of CD66b and low levels of CD62L and CD16 [51, 52, 162, 174]. However, the use of these new markers remains controversial due to lack of clinical validation. Therefore, most of the human cancer studies narrowed their characterisations to evaluate the role of the main subpopulations which are granulocytic (Lin-HLA-DR-CD33+CD11b+CD15+) and monocytic (Lin-HLA-DR-CD14+) [2]. Although CD15 is considered as a marker for granulocytes, this phenotype by itself does not discriminate for MDSCs and this was evident in our analysis when we observed no statistical significant difference in the frequency of Lin-HLA-DR-CD15+ across all study groups. In an attempt to further define this subpopulation, we changed the gating strategy to employ two additional markers CD11b and CD33 subsets which yielded significant difference in the frequency of granulocytic MDSC between PC and CP [162].

Tumour-infiltrating Lin-HLA-DR-CD33+CD11b+CD15+ MDSC subset was significantly expanded in pancreatic tumour in comparison with benign pancreatic tissue. Although, our data have shown no significant expansion of monocytic Lin-HLA-DR-CD14+ in the blood of PC patients when compared with HDs, we observed a 4-fold increase in the frequency of the tumour-
infiltrating subset. This indicates that both MDSC subpopulations have different functions in different human cancers [162].

**4.2.2 Clinical significance of MDSC in PC**

We further explored the clinical significance of circulating MDSCs in PC. We found that the percentage of MDSCs in the peripheral blood of PC patients was not correlated with tumour clinical stage, tumour size or with pre-operative cancer biomarkers CA19-9 and CEA. However, these findings can be skewed by the small study sample and a revalidation study with a larger number of patients is mandatory before these conclusions could be drawn [162].

The heterogeneous characteristics of human MDSCs have limited the number of studies that examined the clinical significance of MDSCs in cancers. We identified the largest six recent studies that examined the diagnostic/prognostic significance of MDSCs in human cancer [49, 53, 151, 175, 176]. The patients’ demographic details and study outcomes for these studies are shown in Tables 9 and 10 respectively [2]. While all studies measured the levels of MDSCs in peripheral blood, only three studies identified their levels in the tumour tissue [53, 176, 179]. Sun et al found that the proportion of MDSCs in CRC tissue was correlated with nodal metastasis, distant metastasis and tumour stage ($P=0.028$) suggesting the involvement of MDSCs in cancer development [176]. However, the authors did not perform any functional assay experiments and did not assess the suppressive features of MDSCs in peripheral blood or tumour tissue. Gros et al characterised the nature and suppressive function of melanoma-infiltrating
MDSCs in addition to the circulating MDSCs [53]. Unexpectedly, the melanoma-infiltrating MDSCs displayed an impaired ability to inhibit \textit{in vitro} T cell proliferation in comparison to circulating MDSCs [2]. This suggested that the tumour-infiltrating MDSCs exhibit less immune suppressive effect than what reported in other studies [164]. In addition and in contrast to the remaining studies [49, 151, 175, 176], Gros \textit{et al} found no significant difference in the percentage and absolute number of 14 different MDSC subsets in the peripheral blood of metastatic melanoma patients when compared to controls [53]. These findings highlight the complex immune regulatory role of tumour specific/derived factors within specific tumour microenvironments and that MDSCs are not universally present in all tumours or they tend to decrease in numbers in advanced stages of cancer [2].

The reports on clinical correlation between circulating/infiltrating MDSC levels and tumour stage and survival are contradicting as shown in Table 10 [2]. There are a number of potential factors to how the controversy exists. These reports included heterogeneous population of diverse cancer types such as breast, lung, melanoma, sarcoma and Gastrointestinal (GI) cancers. In addition, the focus of these reports and many other human studies is only directed towards the characterisation of some MDSC subsets in diverse cancer types, which is likely to lead to an inconclusive characterisation of the suppressive features of MDSCs. The processing methods employed for MDSCs characterisation have also been much variable between studies. The analysis of MDSCs using fresh PBMC or more frequently cryopreserved PBMC offers the advantages of transport and batch analysis which is
essential for multicentre studies [162]. However, in a recent study, the frequency of Lin'HLA-DR'CD15' and Lin'HLA-DR'CD33' subsets were significantly reduced and all other subsets lost their in vitro suppressive activities after cryopreservation for both healthy individuals and cancer patients [177]. Therefore, studies of human MDSCs on fresh PBMCs appear to be a better method than cryopreserved PBMCs while whole blood processing remains the most appropriate methods for complete characterisation of MDSCs. Thus far, the prognostic or predictive significance of MDSCs in human cancer remains inadequately investigated and supported with data from few clinical studies some of which are lacking information on the clinical implications of circulating MDSCs. Similarly, there is only one clinical study that examined the predictive value of MDSC levels for effective immunotherapy in cancer patients [162, 178]. The ratio of DC to MDSC (Lin'HLA-DR'CD33') was measured in patients with advanced kidney cancer (n=20) or melanoma (n=16) who were scheduled to receive a modified high treatment dose of IL-2 [178]. Patients who had more favourable outcomes were found to have had high pre-treatment DC to MDSC ratios, and therefore the levels of circulating MDSCs can predict the likelihood of effective IL-2 treatment [178].

The reports on clinical correlation between circulating/infiltrating MDSC levels and tumour stage and survival are contradicting. In one study, the proportion of MDSCs in CRC tissue was correlated with nodal metastases, distant metastases and tumour stage suggesting the involvement of MDSCs in cancer development [176]. Others found no such correlation between the percentage of MDSCs and cancer clinical stage [151]. The existing
contraversies can be explained by the fact that these reports included heterogeneous population of varied cancer types such as breast, lung, melanoma, sarcoma and gastrointestinal cancers [2, 162]. In addition, the focus of these reports and many other human studies is only directed towards the characterisation of some MDSC subsets in diverse cancer types, which is likely to lead to an inconclusive characterisation of the suppressive features of MDSCs. The processing methods employed for MDSCs characterisation have also varied considerably between studies. Therefore, there is an immense need to validate the prognostic/predictive value of MDSCs in prospective large clinical studies [162].

4.3 Limitation of current study

The findings in this study need to be interpreted in light of the study’s limitations. Our study is limited by the small number of participants; this was largely due to the exclusion of patients with medical problems (e.g. autoimmune diseases) or concurrent inflammation and sepsis as potential confounding factors. The number of tissue samples was also small due to the small percentage of CP and PC patients in whom surgical resection was indicated. It would be advantageous to have a large cohort of tissues including a greater number of CP and PC in order to further the understanding of role of MDSC subsets in pancreatic cancer. Nonetheless, these results together demonstrate, for the first time, the differential levels of MDSCs subsets in pancreatic cancer.

Our flow cytometer configuration only allows to do six colours characterisation and therefore no lineage exclusion was used in Figure 6. As
whole blood contains a lot of neutrophils which express abundant arginase, some one could argue that the ARG1 activity demonstrated in Figure 6 is due to the presence of neutrophils instead of granulocytic MDSC. However, we did not use lineage staining because anti-Lin antibody is FITC and we therefore we cannot use the two FITC antibodies together. Before removing the anti-Lin-FITC from our panel, we checked and did not see any difference in the subpopulations defined whether we include the anti-Lin antibody or exclude it. Additionally, others investigated MDSCs without the lineage exclusion and the most important markers to define MDSC subpopulations are the expressions of CD11b and CD33 and lack of expression of HLA-DR. While it is helpful to check the morphology of MDSC subsets, it is much more important to characterise them using flow cytometry by looking for the expression of different markers.

4.4 Recommendation for further research

The implications of the study have suggested that patients with pancreatic cancer have increased levels of tumour-infiltrating and circulating granulocytic MDSCs. The themes identified in this study provide a context for further research and the current findings could be used to inform the development of new cancer therapeutic targets. It is therefore important to consider further research to understand the exact mechanism on how PC recruits and induces the generation and/or expansion of different subsets of MDSCs as an evasion mechanism from host immune defence. In addition, it would be of great interest to investigate whether MDSC modulate immune responses in cancer patients directly or by activating Treg and whether contact- or cytokine-dependent mechanisms are required for MDSC
generation/expansion by tumour cells, and to determine which chemokines and chemokine receptors are expressed by tumour tissue, Tregs and MDSC and involved in mediating Tregs and MDSC trafficking in the tumour micro-environment. It would of clinical interest to investigate whether the circulating levels of MDSCs can be used as a diagnostic/prognostic marker in patients with PC. However, such study is only potentially achievable in a multicentre multinational setting for sufficient sample size and statistical power.

4.5 Conclusions

In conclusion, we have evaluated the frequency, phenotype, ARG1 expression and clinical significance of MDSCs in PC. We demonstrated a significant increase in circulating and tumour-infiltrating MDSC levels and their functional activity in PC. It is likely that the increased frequency of granulocytic MDSCs in human cancers plays an important role in tumour pathogenesis and progression. Although this finding was not shown in our current study, bigger pre-clinical studies may give a better indication of the diagnostic/prognostic power of MDSCs in PC. Our findings, if incorporated into large prospective validating studies, are of considerable significance for developing new immunotherapeutic strategies via inhibiting and eliminating MDSCs in PC. This work furthers our understanding of the important role of each of these subsets in patients with this cancer and help to identify new therapeutic targets. In the future, pancreatic cancer treatment might be tailored to target the immunosuppressive pathways used by MDSCs [162].
<table>
<thead>
<tr>
<th>Author [Ref No]</th>
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<td>Gabitass et al [151]</td>
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<td>Duffy <em>et al</em> [175]</td>
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<td>Sun <em>et al</em> [176]</td>
<td>49</td>
<td>I</td>
<td>Fresh PBMC &amp; tumour tissue</td>
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<tr>
<td>Zhang <em>et al</em> [179]</td>
<td>64</td>
<td>I</td>
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No: number; AJCC: American joint committee on cancer; PBMC: peripheral blood mononuclear cells
Table 10: Clinical studies of MDSCs in human cancer: diagnostic power and correlation with clinical pathology

<table>
<thead>
<tr>
<th>Author [Ref No]</th>
<th>MDSC subset identified</th>
<th>MDSC level in peripheral blood: cancer vs. normal</th>
<th>MDSC value in tumour</th>
<th>P value</th>
<th>Correlation with clinical features</th>
</tr>
</thead>
</table>
| Diaz-Montero et al [21] | Lin^{low} HLA DR−, CD33+CD11b+          | 2.85 vs. 1.26%                                   | ND                   | $P < 0.0001$ | MDSC percentage and absolute numbers correlate significantly with cancer stage:  
  I/II cancer vs IV cancer:  
  (1.96 vs 3.77%; $P < 0.0001$) (124.1 vs. 260.04 cells/μL; $P < 0.0001$).  
  III cancer vs IV cancer:  
  (2.46 vs 3.77%; $P = 0.014$) (163.7 vs. 260.04 cells/μL; $P = 0.031$). |
<p>| Gabitass et al [151]  | Lin^{low} HLA DR−, CD33+CD11b+          | (2.1, 1.3, 1.5) vs. 0.8%                          | ND                   | $P &lt; 0.001$ | No association between cancer stage and MDSC levels, ($P &lt; 0.53$) but significantly associated with survival ($P &lt; 0.001$). |</p>
<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Description</th>
<th>Comparison</th>
<th>Stat</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>Duffy et al [175]</td>
<td>CD14+HLA-DR-/low</td>
<td>FPBMC 5.5 vs 1.2%, CPBMC 10.03 vs 4.16% whole blood 2.5 vs 0.8%</td>
<td>ND</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Duffy et al [175]</td>
<td>CD15+CD14-</td>
<td>whole blood 59.7 vs 46.9%</td>
<td>ND</td>
<td>$P &lt; 0.003$</td>
</tr>
<tr>
<td>Sun et al [176]</td>
<td>CD33+HLA-DR-</td>
<td>PBMC :1.89 vs 0.54%</td>
<td>2.99 %</td>
<td>$P &lt; 0.05, P &lt; 0.05$</td>
</tr>
<tr>
<td>Zhang et al [179]</td>
<td>Lin−/low HLA DR−, CD33+CD11b+</td>
<td>Whole blood 3.554%; vs 0.818%</td>
<td>4.69%</td>
<td>$P&lt;0.0001$</td>
</tr>
<tr>
<td>Gros et al [53]</td>
<td>CD11b+CD14+</td>
<td>12.6 ± 1.1 vs 13.8 ± 1.2%</td>
<td>49.8 ± 5.3%</td>
<td>$P &lt; 0.48, P &lt; 0.17$</td>
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<tr>
<td>Gros et al [53]</td>
<td>CD11b+ CD14-CD15+</td>
<td>59.2 ± 3.4 vs 52.6 ± 4.2 %</td>
<td></td>
<td>$P &lt; 0.17, P &lt; 0.202$</td>
</tr>
</tbody>
</table>

ND: not determined; MDSC: myeloid derived suppressor cell; PBMC: peripheral blood mononuclear cells; FPBMC: fresh peripheral blood mononuclear cells; CPBMC: cryopreserved peripheral blood mononuclear cells.
Appendices
Appendix 1: Ethical approval

Health Research Authority

NRES Committee North West - Greater Manchester South
Northwest Centre for Research Ethics Committees
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

02 December 2011

Professor Basil Ammori, Consultant Hepatobiliary & Bariatric Surgeon
Department of Surgery
Salford Royal Hospital
Stott Lane
Manchester
M6 8HD

Dear Professor Ammori

Study title: Investigations into the role and function of immunosuppressive cells and macrophage inhibitory cytokine-1 in pancreatic, colorectal cancers and liver metastases

REC reference: 11/NW/0770
Protocol number: BA/EE/YK/11

Thank you for the e-mail from Dr Khaled received on 29 November 2011, responding to the Committee’s request for further information on the above research and submitting revised documentation. This has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned. Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk

A Research Ethics Committee established by the Health Research Authority
Where a NHS organisation’s role in the study is limited to identifying and referring potential participants to research sites (“participant identification centre”), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

Sponsors are not required to notify the Committee of approvals from host organisations

Additional conditions

Please make amendments to the information sheets highlighted in green on the attached sheets.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Please notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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<tr>
<td>GP/Consultant Information Sheets</td>
<td>2</td>
<td>28 November 2011</td>
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<tr>
<td>Investigator CV</td>
<td></td>
<td>20 October 2011</td>
</tr>
<tr>
<td>Letter from Sponsor</td>
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<td>12 October 2011</td>
</tr>
<tr>
<td>Other: CV for Dr Khaled</td>
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<tr>
<td>Other: CV for Dr Elkord</td>
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<td>Other: Letter from NHS Blood and Transplant</td>
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<td>20 October 2011</td>
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</table>

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document “After ethical review – guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:
Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

Sponsors are not required to notify the Committee of approvals from host organisations

**Additional conditions**

Please make amendments to the information sheets highlighted in green on the attached sheets.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Please notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

**Approved documents**

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**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**After ethical review**

**Reporting requirements**

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:
• Notifying substantial amendments
• Adding new sites and investigators
• Notification of serious breaches of the protocol
• Progress and safety reports
• Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/NW/0770 Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project.

Yours sincerely

Dr Ann Wakefield
Chair

Email: elaine.hutchings@northwest.nhs.uk

Enclosure: “After ethical review – guidance for researchers”

Copy to: Linda Kelly, University of Salford

Dr Steve Woby, R&D Department, Royal Oldham Hospital, Pennine Acute Hospitals NHS Trust
Appendix 2: R&D ethical approval

The Pennine Acute Hospitals NHS Trust

Research and Development
Trust Headquarters
North Manchester General Hospital
Delaunays Road
Crumpsall
Manchester
M8 5RB

Dr. Steve Woby – Head of Research & Development
T: 0161 720 2229 / 0779 1458 483
E: steve.woby@pat.nhs.uk

Kat Moklak - R&D Co-ordinator
T: 0161 922 3378
E: kat.moklak@pat.nhs.uk

Dr. Tina Kenny – Deputy Medical Director / Director of Medical and Dental Education
T: 0161 720 5474
E: christina.kenny@pat.nhs.uk

Katie Doyle - R&D Facilitator
T: 0161 604 5233
E: katie.doyle@pat.nhs.uk

Professor Basil Ammori
Consultant Hepatobiliary & Bariatric Surgeon
Department of surgery
Salford Royal Hospital
Stott Lane
Salford
M6 8HD

16th March 2012

Dear Professor Ammori,

Re: Research & Development (R&D) approval (externally sponsored Non CTIMP)

R&D reference number: 11CANCER19
Project title: Investigations into the role and function of immunosuppressive cells and macrophage inhibitory cytokine-1 in pancreatic, colorectal cancers and liver metastases

Ethics number: 11/NW/0770
Site: N.M.G.H

Thank you for providing the Research & Development (R&D) department with your research project information. The above study was considered by The Pennine Acute Hospitals NHS Trust in line with the Research Governance Framework where based on the information provided the impact of the study on the Trust’s resources was reviewed. I am pleased to inform you that the study has received Trust R&D approval.

Following verification of relevant Regulatory approval from The National Research Ethics Service, the following documents as listed on the ethics approval letter have been approved by R&D:

- Ethics approval letters dated 2nd December 2011 and 6th December 2011
- SSI / IRAS application
- Covering letter dated 13th October 2011
- Evidence of insurance or indemnity dated 1st August 2011
- GP / Consultant information sheets (version 2) dated 28th November 2011
- Investigator CV (B. Ammori, Y. Khaled, E. Eikord)
- Letter from Sponsor dated 12th October 2011
- Other: letter from NHS Blood and Transplant dated 14th July 2011
- Protocol (version 1) dated 20th October 2011
- Protocol (version 1) dated 20th October 2011 (signed by PI, B. Ammori)
REC application

Response to request for further information

Participant consent form: patients (version 3) dated 5th December 2011
Participant consent form: controls (version 3) dated 5th December 2011
Participant information sheet: patients (version 3) dated 5th December 2011
Participant information sheet: controls (version 3) dated 5th December 2011
Email from Yazan Khaled confirming study funding, NIHR portfolio status and sample labelling to laboratories dated 15th March 2012

If there are any substantial amendments to the protocol, including the number of patients to be recruited from The Pennine Acute Hospitals NHS Trust, you must obtain a favourable opinion from:

- The National Research Ethics Service
- The Pennine Acute Hospitals NHS Trust (please note that all amendment documentation as approved by The Research Ethics Committee (REC) must be submitted to the R&D department)

On completion of the study you are required to submit a ‘Declaration of End of Study’ form to the main REC, which should also be copied and forwarded to the R&D Department at the above address.
http://www.nres.npsa.nhs.uk/applications/after-ethical-review/endofstudy/

As part of research governance the R&D department is expected to monitor the progress of registered projects. Therefore, on-going projects may be subject to random inspection. Your research must be conducted in compliance with the NHS Research Governance Framework for Health & Social Care.

It is a condition of NHS R&D approval that patient recruitment data should be forwarded on a regular basis. Therefore, project reports must be submitted annually to the main REC and copied to the R&D office until the end of the study.
http://www.nres.npsa.nhs.uk/applications/after-ethical-review/annual-progress-reports/

In addition to your obligations to the study organisers and the REC, the R&D Department must be informed of any governance issues related to the research.

Failure to comply with any of the above may result in withdrawal of approval for the project and the immediate cessation of the research.

Yours sincerely

Dr. Steve Woby
Head of Research & Development

cc: Dr. Yazan Khaled – Clinical Research Fellow
Hepatobiliary Department
North Manchester General Hospital
Deaunays Road
Crumpsall
Manchester
M8 5RB

Mrs Rachel Georgiou - ReGroup Manager
Research & Development
Clinical Sciences Building
Salford Royal NHS Foundation Trust
Salford
Stott Lane
M6 8HD
Study title: Collection of tissues and peripheral blood samples from healthy/chronic pancreatitis individuals for cancer immunotherapy Research

Information Sheet and Consent Forms for Control Subjects

(New Sample)

Invitation:

You are being invited to take part in a research study. Your understanding of the purpose of this research and what your involvement will entail is essential. Please take time to consider being involved with this research by reading the following information sheet and consent form.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

PART 1

This section gives a brief overview of the study to help you decide whether or not you are interested in taking part.

1.1 What is the purpose of the Study

Each year a significant number of people develop pancreatic or colon cancer. We are very keen to find out more about the reasons for this and how we can develop new treatments for cancer. In order to complete the study we need to recruit
healthy individuals like you. The immune system protects the body by identifying and destroying viruses, bacteria and body cells that are potentially cancerous. However, cancer cells send out chemical signals that dampen down the immune system and consequently allow tumours to grow. We wish to conduct a study to investigate the process through which the immune system is suppressed by looking at the nature of the chemical signals sent out by the cancer cells. We will compare your healthy tissue samples to those with cancer. This will help to develop new therapeutic targets for treating cancers. In order to undertake this research work we need healthy tissue samples, blood and biopsies from superficial and deep fat tissue.

1.2 Why have I been chosen?

Although the study aims to evaluate patients who have been diagnosed with cancer of either the pancreas or the colon, we also require “control subjects” who are healthy individuals (like you) undergoing surgery for benign conditions such as laparoscopic cholecystectomy, laparoscopic bariatric surgery or surgery for chronic pancreatitis to participate in order to provide normal tissue samples for comparison with the cancer tissues.

1.3 Do I have to take part?

Participation in this study is entirely voluntary. If you wish to take part, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time without giving a reason. Withdrawal will not affect any care you receive either now or in the future.
1.4 What will happen to me if I take part?
If you agree to take part, we will ask you to gift a small sample of the removed tissue and the surrounding superficial and deep fat tissue for this research and for use in future research. The sample will be removed so that it will not interfere with the routine diagnostic value of the tissue samples. In addition we will ask you to gift 40ml of blood (3 tablespoons) so that we can isolate the cells from it. The sample can be obtained while blood is withdrawn for routine preoperative assessment. No further blood samples will be taken subsequently and no further visits to clinics are needed apart from the ordinary postoperative assessment and follow up. **You will be free to refuse any samples being taken and your decision will not affect your routine patient care/treatment.**

1.5 Will I benefit financially from discoveries using these materials?
No donor will benefit financially from the discoveries of this research. Although discoveries made using these materials may be of commercial value, this will only result from extensive research involving many tumour samples and considerable laboratory and clinical research. Therefore, it is unlikely that any one sample will be critical in this process. You will, however, be informed of such discoveries.

1.6 Are there any drugs or additional test(s) involved?
No. There are no drugs or tests involved.

1.7 Are there any side effects?
There are no side effects other than the slight discomfort, which is normal, during the blood test.

1.8 What are the possible benefits of taking part?

Although there will be no direct benefit to you from this research, such tissue banking and the analysis of the samples will be used to develop new therapies that may be of value to cancer patients in the future.

1.9 Will my taking part in the study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it. The clinical research fellows at North Manchester General Hospital will collect information from the hospital notes about your medical history. This information will be put into an anonymous form for future use and your personal details will not be available to the laboratory researchers or others outside the hospital. Occasionally we may share only the clinical data with other health organisations in the UK and within the European Union without disclosing any identifying personal details.

1.10 Who is organising and funding the research?

This is a research project run by NHS consultants and research fellows and supported and paid for by charitable research funds with the NHS and the University of Salford. The medical team involved in your treatment and care will receive no payment because of your involvement in this study.
PART 2

**Study title:** Collection of tissues and peripheral blood samples from healthy individuals for cancer immunotherapy Research

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

2.1 What will happen if I don’t want to carry on with the study?

You are free to withdraw from the study at any time, simply by telling a researcher your wishes. You do not have to give a reason. If you withdraw completely from the study, we still need to use the data collected before your withdrawal.

2.2 Will my GP be informed?

A standard letter will be sent to your GP informing him that you are participating in this study.

2.3 What will happen to any samples I give?

Your samples will be transported by a suitably qualified person to the University of Salford laboratories for examination and analysis. We would like to store the samples and consider using them for future research studies as appropriate. All samples will be stored at Salford Royal Foundation Trust in Manchester. Occasionally, samples are sent to the other laboratories inside the UK and used as part of this Project or future research studies, without disclosing your identity.

2.4 Will the material be used for other purposes?

The aim of the research is to discover new molecules that can be targeted in treatments but secondary discoveries are possible. If anything of direct clinical relevance to you were to be discovered the information would be fed back to you. The
use of tissue materials for other types of research cannot be anticipated at present. However, if such research is planned the Local Ethics committee will be consulted. You can tell us today whether you would like to gift your tissue samples to be used for any other relevant research in the future by signing the consent form.

2.5 What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [Dr. Yazan Khaled, Mr. Basil Ammori]. If you remain unhappy and wish to complain formally, you can do this by contacting Patient Advice and Liaison Service (PALS) calling Tel - 0161 720 2707 between 9.30 am and 4.30 pm or emailing PALS.Northmanchester@pat.nhs.uk or refer to North Manchester Complaints Advice.

In the event that something does go wrong and you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against The NMGH NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

2.6 Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the National Research Ethics Service Committee in North West- Greater Manchester South.
2.7 How can I get further information?

This study is being organised by Prof. Basil Ammori and Dr. Eyad Elkord. Both or any of their research fellows (all can be contacted through the research team office at North Manchester General Hospital on 0161 9184227) will be happy to provide further information.
Appendix 4: Patient information sheet for cancer patients

Study title: Collection of tissues and peripheral blood samples from pancreatic or colorectal cancer patients for cancer immunotherapy Research

Patient Information and Consent
(New Sample)

Invitation:

You are being invited to take part in a research study. Your understanding of the purpose of this research and what your involvement will entail is essential. Please take time to consider being involved with this research by reading the following information sheet and consent form.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

PART 1

This section gives a brief overview of the study to help you decide whether or not you are interested in taking part.

1.10 What is the purpose of the Study

Each year a significant number of people develop pancreatic or colon cancer. We are very keen to find out more about the reasons for this and how we can develop new treatments for cancer. The immune system protects the body by identifying and destroying viruses, bacteria and body cells that are potentially cancerous. However, cancer cells send out chemical signals that dampen down the immune system and
consequently allow tumours to grow. We wish to conduct a study to investigate the processes through which the immune system is suppressed and examine the nature of the chemical signals sent out by the cancer cells. This will help develop new therapeutic targets for treating cancers. In order to undertake this research work we need samples of tumour cells, blood and biopsies from superficial and deep fat tissue from cancer patients.

1.11 Why have I been chosen?

We are asking people who have been diagnosed with, or are suspected of having cancer of either the pancreas or the colon for which they are about to undergo a biopsy or surgery to remove all or part of it. We hope that 400 people will take part in this study.

1.12 Do I have to take part?

Participation in this study is entirely voluntary. If you wish to take part, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time without giving a reason. Withdrawal will not affect any care you receive either now or in the future.

1.13 What will happen to me if I take part

If you agree to take part, we will ask you to gift a small sample of the tumour and the surrounding superficial and deep fat tissue for this research and for use in future research. The sample will be removed so that it will not interfere with the routine diagnostic value of the tumour. In addition we will ask you to gift 40ml of blood (3 tablespoons) so that we can isolate the cells from it. The sample can be obtained while blood is withdrawn for routine preoperative assessment. No further blood
samples will be taken subsequently and no further visits to clinics are needed apart from the ordinary postoperative assessment and follow up. You will be free to refuse any samples being taken and your decision will not affect your routine patient care/treatment.

1.14 Will I benefit financially from discoveries using these materials?
No donor will benefit financially from the discoveries of this research. Although discoveries made using these materials may be of commercial value, this will only result from extensive research involving many tumour samples and considerable laboratory and clinical research. Therefore, it is unlikely that any one sample will be critical in this process. You will, however, be informed of such discoveries.

1.15 Are there any drugs or additional test(s) involved?
No. There are no drugs or tests involved.

1.16 Are there any side effects?
There are no side effects other than the slight discomfort, which is normal, during the blood test.

1.17 What are the possible benefits of taking part?
Although there will be no direct benefit to you from this research, such tissue banking and the analysis of the samples will be used to develop new therapies that may be of value to you or other cancer patients in the future.

1.18 Will my taking part in the study be kept confidential?
Yes. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the
hospital/surgery will have your name and address removed so that you cannot be recognised from it. The clinical research fellows at North Manchester General Hospital will collect information from the hospital notes about your medical history and about your tumour. This information will be put into an anonymous form for future use and your personal details will not be available to the laboratory researchers or others outside the hospital. Occasionally we may share only the clinical data with other health organisations in the UK and within the European Union without disclosing any indentifying personal details.

1.10 Who is organising and funding the research?

This is a research project run by NHS consultants and research fellows and supported and paid for by charitable research funds with the NHS and the University of Salford. The medical team involved in your treatment and care will receive no payment because of your involvement in this study.

PART 2

Study title: Collection of tissues and peripheral blood samples from pancreatic or colorectal cancer patients for cancer immunotherapy Research

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.
2.6 What will happen if I don't want to carry on with the study?
You are free to withdraw from the study at any time, simply by telling a researcher your wishes. You do not have to give a reason. If you withdraw completely from the study, we still need to use the data collected before your withdrawal.

2.7 Will my GP be informed?
A standard letter will be sent to your GP informing him that you are participating in this study.

2.8 What will happen to any samples I give?
Your samples will be transported by a suitably qualified person to the University of Salford laboratories for examination and analysis. We would like to store the samples and consider using them for future research studies as appropriate. All samples will be stored at Salford Royal Foundation Trust in Manchester. Occasionally, samples are sent to the other laboratories inside the UK and used as part of this Project or future research studies, without disclosing your identity.

2.9 Will the material be used for other purposes?
The aim of the research is to discover new molecules that can be targeted in treatments but secondary discoveries are possible. The use of tissue materials for other types of research cannot be anticipated at present. However, if such research is planned the Local Ethics committee will be consulted. You can tell us today whether you would like your gifted tissue samples to be used for any other relevant research in the future by signing the consent form.

2.10 What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [Dr. Yazan Khaled, Mr. Basil Ammori]. If you remain unhappy and wish to complain formally, you can do this by contacting Patient Advice and Liaison Service (PALS) calling Tel - 0161 720 2707 between 9.30 am and 4.30 pm or emailing PALS.Northmanchester@pat.nhs.uk or refer to North Manchester Complaints Advice.

In the event that something does go wrong and you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against The NMGH NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

2.6 Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the National Research Ethics Service Committee in North West-Greater Manchester South.

2.11 How can I get further information?

This study is being organised by [Mr. Basil Ammori and Dr. Eyad Elkord]. Both Mr. Ammori and Dr. Elkord or any of their research fellows (all can be contacted through the research team office at North Manchester General Hospital on 0161 9184227) will be happy to provide further information.
Appendix 5: Consent form

Study title: Collection of tissues and peripheral blood samples from pancreatic or colorectal healthy individuals for cancer immunotherapy Research (fresh samples)

Please initial boxes

1. I confirm that I have read and understand the dated information sheet attached

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason, without my medical care or legal rights affected.

3. I am willing to allow access to my medical records but understand that strict confidentiality will be maintained. The purpose of this is to check that the study is being carried out correctly.

4. I give permission for the researchers to treat my blood and tissue samples as a gift and I am happy for it to be used in future, ethically approved research projects.

5. I understand that blood and tissue I provide will be stored until they no longer hold any merit for research after which they will be disposed of lawfully. I have indicated any specific requests for the disposal of the samples below.

6. I consent to export of anonymised data and samples to researchers inside and outside the UK.

7. I agree to my GP being informed of my participation in the study.

8. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test.
9. I give permission that if a new test or treatments arise based on findings discovered in my samples, commercial companies may have access to my samples in the future as necessary, and patents may be taken out. I understand that I will not benefit financially, but that a proportion of any profits may go towards further research.

10. I agree to take part in the above study.

_________________________  ______  ______________________
Name of patient             Date                  Signature

_________________________  ______  ______________________
Name of Person taking consent Date                  Signature
(if different from researcher)

_________________________  ______  ______________________
Researcher                 Date                  Signature
Bibliography:


patients are Stat3hi and overexpress CD80, CD83, and DC-sign. Cancer research. 70:4335-4345.


96. Veltman JD, Lambers MEH, van Nimwegen M, Hendriks RW, Hoogsteden HC, Aerts JGJV, Hegmans JPJJ (2010) COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived


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