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Pro-inflammatory and tumour proliferative properties of excess visceral adipose tissue

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Abbreviations: vACM: visceral adipose tissue conditioned media. sACM: subcutaneous adipose tissue conditioned media, CT: computed tomography, VEGF: Vascular endothelial growth factor, IL: Interleukin, NK: Natural Killer, iNKT: invariant Natural Killer T cell.

Abstract

Obesity has been associated with increased incidence and mortality of oesophageal and colorectal adenocarcinoma. Excess central adiposity may drive this association through an altered inflammatory milieu. Utilizing a unique adipose tissue bioresource we aimed to determine the pro-tumour properties of visceral adipose tissue. Comparing subcutaneous and visceral adipose tissue depots, we observed significantly higher levels of VEGF and IL-6, along with significantly higher proportions of CD8$^+$ T cells and NKT cells in visceral adipose tissue. Significantly higher levels of VEGF were observed in the conditioned media from visceral adipose tissue of centrally obese compared to non-obese patients. We also report a significant increase in oesophageal and colorectal tumour cell proliferation following culture with conditioned media from visceral adipose tissue of centrally obese patients. Neutralizing VEGF in the conditioned media significantly decreased tumour cell proliferation. This novel report highlights a potential mechanism whereby visceral adipose tissue from centrally obese cancer patients may drive tumour progression.

1. Introduction

Epidemiological studies have established a significant association between obesity and numerous co-morbidities including diabetes mellitus, cardiovascular disease and cancer [1, 2]. We and others have shown that oesophageal adenocarcinoma and colorectal cancer, which arise in a background of chronic inflammation, are strongly linked with obesity [3, 4], making them ideal models to study the mechanisms driving obesity-associated malignancies. A number of large scale studies have demonstrated not only an increase in cancer incidence, but also an rise in cancer mortality with excess body weight [5-7]. However, the mechanisms underlying these associations and the contribution of individual adipose tissue depots remain poorly understood.

Adipose tissue is a multifunctional organ, secreting a variety of adipokines and cytokines, which can regulate physiological functions systemically through endocrine-like activity [8]. These factors include adiponectin, leptin, and numerous cytokines such as TNF-alpha, IL-6, monocyte chemoattractant protein-1 (MCP-1), IL-8, IL-10 and VEGF [9]. Many of these factors have been implicated in promoting tumour development and progression [10]. The altered adipocytokine and growth factor profile in centrally obese patients is believed to be an important contributing factor to the pathogenesis of many co-morbidities associated with obesity, including cancer. Subcutaneous and visceral adipose tissue depots are increasingly recognized as pathologically distinct, with visceral adipose tissue described as being more metabolically active [11]. Circulating levels of pro-inflammatory adipokines such as leptin, have been shown to correlate with visceral adipose tissue. Both *in vitro* and *in vivo* studies confirm leptin promotes cellular proliferation, angiogenesis and metalloproteinase expression in both oesophageal and colon cancer [12, 13].

Obesity, particularly central obesity, results in the establishment of a state of chronic systemic low-grade inflammation associated with pro-tumourigenic and pro-angiogenic factors along with enhanced genetic instability [14]. Obesity-associated inflammation is strongly influenced by immune cell populations present within the adipose tissue. Analysis of the cellular content and inflammatory profile of adipose tissue depots will ultimately enhance the understanding of biological pathways linking obesity with cancer.

In our academic medical centre, an adipose tissue bioresource was established to enable research into the mechanisms linking excess visceral adiposity and gastrointestinal cancer. This novel study, in a cohort of gastrointestinal cancer patients, aimed to firstly determine the pro-inflammatory and pro-tumourigenic properties of subcutaneous and visceral adipose tissue, and secondly the impact of central obesity on these pathways.

2. Materials and Methods

2.1 Patient Recruitment, Anthropometry and Metabolic screening

All patients undergoing elective abdominal surgery within our unit for resectional surgery were invited to partake in the adipose tissue biobank. Only individuals with no previous history of cancer prior to their current diagnosis were recruited and informed consent sought. Ethical approval was granted from the St. James's Hospital Ethics review board for sample collection for adipose tissue research.

Body weight, height, central waist circumference and blood pressure were measured for each patient. The International Diabetes Federation waist circumference cut-offs for European patients were used to determine obesity status by waist circumference, >80cm for females and >94cm for males [15]. The metabolic syndrome, defined as a cluster of metabolic disturbances including raised triglycerides and fasting plasma glucose, reduced HDL-cholesterol, raised blood pressure and visceral obesity was assessed in all patients along with blood pressure measurements [15]. The visceral and subcutaneous adipose tissue area (VFA & SFA) was calculated in patients who underwent abdominal CT scan, with all measurements performed by a single experienced radiologist. The cross-sectional surface area of the subcutaneous and visceral fat compartment at the level of the inter-vertebral disc between L3 and L4 was calculated, using a previously standardized and validated technique [16]. Briefly, subcutaneous and visceral compartments were delineated and a density contour software program then measured all pixels within the two compartments with the same density (or "attenuation number" as fat tissue [between -150 and -50 Hounsfield units]). This was then converted and expressed as a cross-sectional area in cm^2. Central or visceral obesity is defined as having a visceral fat area exceeding $130cm^2$ [16]. All centrally obese patients in this study had a VFA $>130cm^2$.

2.2 Adipose Tissue Processing

At the commencement of surgery, matched deep subcutaneous and greater omental adipose tissue samples were obtained from each patient, representative of peripheral and visceral adipose tissue respectively. The participating surgeons avoided the use of diathermy when excising the adipose tissue and the use of scalpel and hand ties minimized the risk of tissue damage. Adipose tissue was immediately placed into sterile adipose transport buffer (PBS, 5.5mM glucose and 50 µg/ml gentamicin) prior to processing. To prepare adipose conditioned media (ACM), an adapted protocol from Fried *et al*. was used [17]. Briefly, the adipose tissue was finely minced into 20–50 mg pieces, washed in sterile PBS to remove excess blood. 5g of adipose tissue was cultured in 10mls of M199 (Biosciences; Dublin, Ireland) cell culture medium supplemented with 50 µg/ml gentamicin for 72 hours. Peripheral blood for serum and cellular analysis was also obtained from each patient on the day of but prior to surgery.

2.3 RNA Extraction

RNA was extracted from the adipose tissue using Qiagen RNeasy Lipid Tissue mini-kits according to manufacturer's instructions (Qiagen, Sussex, U.K.). Briefly, tissue samples were first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer and the sample was then applied to an RNeasy mini spin column where the total RNA binds to the membrane and contaminants are efficiently washed away. Purity and yield of extracted RNA was assessed using the nanodrop spectrophotometer ND 1000 (Delaware, USA). A number of different adipose tissue storage media were assessed to determine which gave the highest quality and yield of RNA, including RNAlater (Qiagen, Sussex, U.K.), Tri-reagent (Sigma Aldrich, Co. Wicklow, Ireland), adipose tissue transport buffer and immediate snap freezing. In addition, RNA integrity from whole snap frozen adipose tissue was assessed using Agilent 2100 Bioanalyser, a 'lab-on-a-chip' micro-fluidics based platform. An electrophoretic trace of the RNA sample is produced, including any degradation products which may be present, in order to generate an RNA integrity number (RIN). Samples with a RIN value >7 were deemed acceptable for use.

2.4 Real-time PCR

cDNA was synthesized using 1µg RNA from adipose tissue samples which was added to 1µL of random primers (Promega, WI, USA) and made up to a volume of 12µL with RNase-free H2O, incubated for 10 mins at 70°C and placed on ice for 1 min. 8 µL of a reverse transcription master mix (0.5 µL dH2O, 0.5 µL RNase inhibitor (1 Unit/µl), 0.5 µL 10mM dNTPs, 0.5 µL SuperScript III reverse transcriptase (200 Units/µl), 4 µL 5X RT Buffer (250 mM Tris-HCl (pH 7.5), 2 µL 0.1mM dTT, all reverse transcription reagents were purchased from Invitrogen Corporation, CA, USA), was added to the samples and placed in a thermal cycler for 60 min at 37°C.

Taqman fluorogenic gene expression probe sets (Applied Biosystems, Courtaboeuf, France) were used for all gene expression experiments. All probe sets used were FAM labelled: IL-6 Assay ID: Hs00985639_m1: IL-8 Assay ID: Hs99999034_m1: VEGF Assay ID: Hs00900055_m1: TNF Assay ID: Hs00174128_m1: Leptin Assay ID: Hs00174877_m1: Adiponectin Assay ID: Hs00605917. Quantitative normalisation of cDNA in each sample was performed using the ribosomal RNA subunit 18s (Assay ID: Hs 99999901_s1) as an endogenous control. Real time PCR was performed using an ABI Prism 7900 HT Fast Real-Time PCR system (Applied Biosystems, Courtaboeuf, France). Thermal cycling conditions were as follows: Step 1 – 2min at 50°C, Step 2 – 10 min at 95°C, Step 3 – 15 sec at 95°C and 1 min at 60°C repeated for 40 cycles. Relative quantification of gene expression was examined using the comparative cycle threshold (CT) method.

2.5 Serum and ACM Protein Quantification

Enzyme-linked immunosorbent assay (ELISA) DuoSet assays (R&D systems, Inc., MN, USA) were used to assess IL-6 (DY206), IL-8 (DY208), VEGF (DY293B), TNF-α (DY210), Leptin (DY398) and Adiponectin (DY1065) in serum and ACM. ELISAs were carried out according to the manufacturer's instructions, briefly, high binding ELISA plates (Greiner Bio-One) were coated with capture antibody overnight prior to blocking with 5% milk. A serial dilution was made of the relevant recombinant protein to generate an 8 point standard. Following the addition of a detection antibody, streptavidin-HRP was added to each well, followed by the addition of the substrate tetramethylbenzidine (TMB) (Sigma Aldrich, Wicklow, Ireland) until the colour developed. The reaction was stopped by the addition of IM H_2SO_4 and the plate was read immediately at 450 nm using a VersaMax microplate reader (Molecular Devices, CA, USA). Analyte concentrations were determined using the standard curve.

2.6 Enzymatic digestion of adipose tissue for flow cytometry

Adipose tissue was enzymatically digested to separate adipocytes and the stromal vascular fraction (SVF), in collagenase buffer (2mg/ml collagenase type II, 4% (w/v) bovine serum albumin in Kreb's

Ringer bicarbonate buffer (118 mM NaCl, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 1.25 mM $CaCl_2$, 1.2mM $MgSO_4$, 10 mM HEPES, pH 7.4)). The tissue was incubated for 1 hour on a shaking incubator at 37˚C at 180 rpm, after which time the sample was passed through a 500 μm polypropylene filter (BD Biosciences; CA, U.S.). Floating adipocytes were removed and the remaining erythrocytes were lysed. The cells were resuspended in FACS buffer (PBS (Lonza, Wokingham, UK), 2% Fetal calf serum (Lonza, Wokingham, UK), 0.1% NaN_3), counted and assessed for viability using trypan blue dye exclusion. Cells were incubated with commercially available anti-human antibodies or appropriate isotype control antibodies (CD3 PE, CD56 FITC, CD19 PE and CD11c APC from BD Pharmingen, San Diego, CA, BDCA-1 FITC and BDCA-2 PE from Miltenyi Biotech (Bergisch Gladbach, Germany), CD4 Tri-color and CD8 Tri-color from Caltag Medsystems (Buckingham, UK) or CD14 APC from eBiosciences (San Diego, CA). Flow cytometry was performed using a FACSCalibur™ flow cytometer (Becton-Dickson, San Jose, CA) with CELLQuest™ software. Propidium iodide staining was used to exclude dead cells and a minimum of 10^4 events were collected in the lymphocyte or monocyte gate as appropriate for each sample.

2.7 BrdU Proliferation assays

OE33 oesophageal adenocarcinoma and HCT-15 colorectal cancer cells were maintained in culture in RPMI 1640, supplemented with 10% fetal bovine serum (Life Technologies, Inc), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco-BRL, Paisley, UK). Experiments were performed when cells were approximately 80% confluent and were set up at 5 x10^3 cells per well in a 96 well plate. Cells were allowed to adhere and were then serum starved for 24 hrs. Media was removed and cells were cultured with 100 μl serum-free control M199 media or ACM for a further 24 hrs. ACM was generated using either visceral or subcutaneous adipose tissue from both obese and non-obese patients. Alternatively, a range of concentrations of recombinant IL-6 (0.001 – 10 ng/ml) or VEGF (0.1 – 1000 ng/ml), or neutralizing antibody to VEGF (1μg/ml) or IL-6 (1μg/ml) was added. BrdU incorporation was assessed according to manufacturer's instructions (Roche, Hertfordshire, UK). The plate was read immediately at 450 nm using a VersaMax microplate reader (Molecular Devices, CA, USA). Cells cultured with M199 media alone were used as controls and baseline proliferation was set at 100%.

2.8 Statistical Analysis

Statistical Analysis was performed using GraphPad InStat3 and Prism5. Data was expressed as mean ± s.e.m. Students *t*-test was used to compare the mean values between two groups. P values less than 0.05 were considered significant.

3. Results

3.1 Patient demographics

Biospecimens were obtained from 281 patients undergoing surgery for gastrointestinal cancer (Table I). Patients had a mean age of 66.4 years, and 66% were male. Over one third of patients (36.6%) met the International Diabetes Federation definition for the Metabolic syndrome [15]. Approximately 55% of these patients were centrally obese by waist circumference (>80cm females, >94cm males) and CT measurements (VFA > 130cm^3), while just over 16% were deemed to be obese by BMI (Table 1). This observation underlines the importance of adequate assessment of obesity status in patients, particularly central obesity, which is linked to metabolic abnormalities and obesity associated comorbidities.

3.2 VEGF, IL-6 and leptin gene expression is significantly higher in visceral compared with subcutaneous adipose tissue.

As adipose tissue has notoriously low RNA yield, the quality and yield of RNA from adipose tissue was assessed over time following snap freezing in liquid nitrogen, as this storage method gave the greatest RNA yield and purity. Tissue samples were stored at -80^{o}C for 1 month, 6 months, 1 year and 2 years were processed for RNA extraction. RNA yield was higher from visceral adipose tissue compared with subcutaneous adipose tissue at all time points. The average yield from 100mg of visceral adipose tissue was approximately 5.4 μg, while the average yield from subcutaneous adipose tissue was approximately 2.46 μg. A slight decrease in RNA yield was observed following prolonged storage of adipose tissue up to 2 years of 7% and 3% in visceral and subcutaneous tissue depots respectively. The purity of RNA, assessed by the A260/280 and A260/230 ratios, was maintained over this period.

Real Time qPCR was performed for a range of relevant adipocytokines including IL-8, VEGF, TNF-alpha, IL-6, adiponectin and leptin. All adipocytokines were expressed at the mRNA level in all subcutaneous and visceral adipose tissue analysed. Relative to subcutaneous adipose tissue, mRNA levels of VEGF ($p<0.01$), IL-6 ($p<0.05$) and leptin ($p<0.001$) were significantly elevated in visceral adipose tissue (n=25 patients). Similar gene expression levels were observed for IL-8, TNF-α and adiponectin between subcutaneous and visceral adipose tissue depots (Figure 1).

3.3 VEGF and IL-6 protein was significantly higher in ACM from visceral compared with subcutaneous adipose tissue.

Protein concentrations of IL-8, VEGF, TNF-α, IL-6, adiponectin and leptin was assessed using commercially available ELISAs in the serum and ACM generated from subcutaneous (sACM) and visceral (vACM) adipose tissue (n=45 matched patients). Similar to the gene expression analysis, significantly higher levels of IL-6 ($p=0.03$) and VEGF ($p<0.0001$) were observed in the vACM compared with matched patient sACM (Figure 2A and 2B). Elevated leptin ($p=0.3277$) and decreased TNF-α ($p=0.0527$) was also observed in vACM compared with sACM, but this did not reach significance (Figure 2A and 2B).

3.4 Significantly higher proportions of NKT and $CD8^+$ T cells are present in visceral adipose tissue compared with subcutaneous adipose tissue.

Immunophenotyping of both subcutaneous and visceral adipose tissue, as well as peripheral blood, was carried out by multiparameter flow cytometry, examining the presence of myeloid and plasmacytoid derived dendritic cells (DC), monocytes, natural killer cells (NK), natural killer T cells (NKT), B lymphocytes, $CD4^+$ and $CD8^+$ T cells. Comparing subcutaneous and visceral adipose tissue, a significantly higher proportion of both NKT ($p<0.05$) and $CD8^+$ T cell ($p<0.05$) was observed in the visceral adipose tissue (Figure 3). No significant differences in the proportions of myeloid DC ($p=0.722$), plasmacytoid B lymphocytes ($p=0.561$), $CD4^+$ T cells ($p=0.363$), $CD14^+$ monocytes ($p=0.635$) or NK cells ($p=0.370$) were observed in visceral compared with subcutaneous adipose tissue. (Figure 3).

3.5 VEGF is significantly elevated in the serum and visceral adipose tissue of centrally obese patients.

To determine the influence of visceral adipose tissue on adipocytokine production in the circulation of these cancer patients, VEGF, IL-6, leptin and adiponectin levels were assessed in the serum of normal weight patients (n=18) and centrally obese patients (n=27). VEGF ($p<0.05$) and leptin ($p<0.01$) protein concentrations were significantly higher, while adiponectin ($p<0.05$) was significantly lower in the serum of centrally obese patients compared with normal weight patients (Figure 4A). IL-6 was also higher in the serum of centrally obese patients but this did not reach significance ($p=0.2183$).

Comparison of vACM from non-obese and centrally obese patients revealed significantly higher VEGF ($p<0.01$) and significantly lower adiponectin ($p<0.05$) in the vACM from centrally obese patients. No significant difference was observed in IL-6 ($p=0.947$) or leptin ($p=0.662$) levels in the vACM (Figure 4B).

3.6 ACM from centrally obese patients induced significantly more proliferation than ACM from non-obese patients, in both oesophageal and colorectal cancer cells.

OE33 (oesophageal adenocarcinoma) and HCT-15 (colorectal cancer) cells were cultured for 24 hours in the presence of either sACM or vACM generated from either centrally obese or non-obese patients compared with media alone. BrdU incorporation assay was used to assess cellular proliferation. Significantly higher levels of cellular proliferation were observed following culture with vACM from centrally obese patients ($p<0.05$) in both oesophageal adenocarcinoma and colorectal cancer cells. Overall, higher levels of proliferation were observed following culture of cancer cells with vACM compared with sACM (Figure 5). No significant increase in proliferation of OE33 or HCT-15 cells was observed following culture with sACM or vACM from non-obese patients over that of media alone ($p>0.1$ for all comparisons).

3.7 Treatment of OE33 and HCT-15 cells with recombinant IL-6 and VEGF results in dose dependent increase in cellular proliferation, while neutralizing VEGF significantly reduces cellular proliferation.

Both OE33 and HCT-15 cells were treated with increasing doses of recombinant human IL-6 (0.001 – 10 ng/ml) or VEGF (0.1 - 1000 ng/ml) for 24 hours. After which time a BrdU proliferation assay was performed. A significant increase ($p<0.05$) in both OE33 and HCT-15 proliferation was observed at concentrations greater than 1 ng/ml of IL-6 (Figure 6A). Similarly, a significant increase in OE33 and HCT-15 cell proliferation was observed following culture with VEGF at concentrations greater than 100 ng/ml for HCT-15 and 1μg/ml for OE33 (Figure 6A). Neutralizing antibodies at concentrations based on their ND_{50} were used to specifically deplete VEGF or IL-6 from the vACM of centrally obese patients, in order to determine their contribution to tumour cell proliferation. Both OE33 and HCT-15 cells were treated with M199 media alone as the control, vACM or vACM treated with neutralizing antibodies for 24 hours. After which time a BrdU assay was performed. Neutralizing IL-6 did not significantly ($p>0.2$) alter tumour cell proliferation (Figure 6B). Neutralizing VEGF resulted in a significant decrease ($p<0.05$) of both OE33 and HCT-15 tumour cell proliferation, suggesting that it is a critical component of visceral adipose tissue secretome driving the tumourigenic process in centrally obese patients (Figure 6B).

3.8 vACM from centrally obese cancer patients induces significantly more tumour cell proliferation than that of non-obese and non-cancer patients.

OE33 and HCT-15 cells were cultured in the presence of vACM generated from centrally obese and non-obese cancer or non-cancer patients. BrdU incorporation assay was used to assess cellular proliferation. Significantly higher levels of cellular proliferation were observed following culture with vACM generated from centrally obese cancer patients ($p<0.05$) in both oesophageal adenocarcinoma and colorectal cancer cells (Figure 7). No significant increase in proliferation of tumour cells was observed following culture with vACM from non-obese patients (Figure 7).

4. Discussion

Obesity has increased markedly over the past two decades and is a well established risk factor for the incidence of both oesophageal adenocarcinoma and colorectal cancer [4]. Furthermore, obesity is associated with increased rates of cancer mortality [18]. Previous studies pertaining to the epidemiological association between obesity and cancer are predominantly based on the crude measure of body fatness, body mass index (BMI). More appropriate measures of central obesity include waist circumference or CT determined fat area, which enables accurate quantification of subcutaneous and visceral adipose tissue depots. A more detailed understanding of the role of individual adipose tissue compartments in the pathogenesis of obesity-related cancer is required to uncover pathways and develop new targets for therapy.

Adipose tissue is now recognised as endocrine and paracrine organ, whose function impacts on numerous physiological processes including inflammation, energy homeostasis, lipid and glucose metabolism [19, 20]. To characterize the association between visceral adiposity and gastrointestinal cancers at a molecular and cellular level, an adipose tissue bioresource was established within our unit. Within this large bioresource just 16% of patients were classified as obese according to BMI, in contrast to 56% and 55% classified as centrally obese by waist circumference and visceral fat area by CT respectively. This supports previous studies that suggest waist-circumference and visceral fat area perform better than BMI as predictors of cancer development and underline the importance of adequate assessment of obesity status in patients [21, 22]. Indeed, epidemiological associations of obesity and cancer may be grossly underestimated, as many of these studies use BMI as the measure of obesity status.

Subcutaneous and visceral adipose tissue depots are pathologically and immunological distinct [23]. Therefore, depot-specific differences in gene and proteome expression profiles may contribute to disorders linked to visceral obesity. Gene expression analysis in paired visceral (omentum) and abdominal subcutaneous tissue from gastrointestinal cancer patients, revealed a 4.5 fold higher expression of leptin in visceral compared to subcutaneous adipose tissue. Despite the fact that leptin protein concentrations in the vACM were higher than that of sACM, this did not reach significance. This may be due to the fact that gene expression analysis was carried out using the adipose tissue, whereas the ELISAs measured the secreted protein in the ACM. Additionally, post-translational modifications of the gene may regulate subsequent secretion of the protein.

Leptin expression is thought to be influenced by pro-inflammatory cytokines and studies have reported circulating levels to correlate solely with omental mRNA expression [24]. Other studies have shown that circulating leptin levels are directly correlated with adipose tissue mass and increased concentrations, as we observed in this study, result from both increased fat mass and increased release from larger adipocytes [25]. Evidence suggests that leptin has several actions, including stimulation of tumour cell growth, migration and invasion, and enhancement of angiogenesis, which may all play a role in tumour development and progression [25].

This current study supports previous reports that visceral adipose tissue expression of IL-6 and VEGF mRNA is greater than matched subcutaneous adipose tissue [26]. Moreover, vACM contained significantly higher levels of both IL-6 and VEGF ($p<0.05$) compared with sACM. VEGF is a known pro-angiogenic and survival factor for a variety of solid malignancies and its expression in visceral adipose tissue may have a local effect on cancer development [27]. The omentum has been reported to be a site for the seeding and growth of peritoneal metastasis from both ovarian and endometrial cancer [28, 29]. Our data would suggest that this process may be facilitated at least in part by the enhanced expression of pro-tumor cytokines such as IL-6 and VEGF in the omentum of centrally obese patients.

Circulating concentrations of pro-inflammatory cytokines have been shown to directly relate to the degree of obesity in humans. In our study both leptin and VEGF, were both significantly elevated and adiponectin was significantly decreased in the serum of cancer patients classified as centrally obese compared with normal weight cancer patients. Circulating levels of leptin correlate with adiposity, and studies confirm leptin promotes cellular proliferation and angiogenesis in gastro-intestinal cancer [25]. These data would suggest that in addition to a potential local effect in the omentum, adipose tissue accumulation in the visceral cavity may regulate cancer development and growth at distant sites through paracrine mechanisms. Circulating levels of VEGF have previously been shown to correlate with central adiposity as opposed to subcutaneous fat in non-cancer cohorts [30]. Adiponectin is the most abundant adipocytokine and consistent with our findings, its level is inversely correlated with obesity [31]. It functions as an anti-angiogenic and anti-inflammatory cytokine. It has also been shown to inhibit tumour growth in animals models [31].

Studies on the immune properties of adipose tissue have largely been conducted in animal experimental models, with little research in humans. This novel study compared the innate and adaptive immune cell subsets within visceral and subcutaneous adipose depots, which may be a potential source of pro-tumourigenic factors. A significantly higher proportion of both NKT and CD8$^+$ T cells were observed in visceral compared to subcutaneous adipose tissue, in this cohort of gastrointestinal cancer patients. A study by Lynch *et al*., also demonstrated that the omentum is a particularly rich source of invariant NKT (iNKT) cells in humans, however, iNKT-cell frequencies were significantly lower in patients with severe obesity and patients with colorectal carcinoma compared with lean healthy subjects [32]. However, a study by Duffaut *et al*., examining T cell infiltration in matched human subcutaneous and visceral adipose tissue demonstrated a significant increase in both CD4$^+$ and CD8$^+$ T cells in visceral compared with subcutaneous adipose tissue in healthy women [33]. While a significant increase in CD8$^+$ T cell frequency was observed in this study, no significant increase was observed in the CD4$^+$ T cell population, possibly suggesting an altered immune cell profile in visceral adipose tissue of cancer patients.

To our knowledge, this is the first study to report that vACM of centrally obese cancer patients significantly induced proliferation of both oesophageal and colorectal tumour cells. vACM or sACM from non-centrally obese patients did not affect tumour cell proliferation. Notably, similar to the results observed in the circulation, VEGF production in vACM from centrally obese patients was significantly higher than non-obese patients. Lower adiponectin was also observed in the vACM of centrally obese patients. These findings could suggest that these factors may, at least in part, be driving the proliferation of tumour cells. Similar to other studies which have examined the impact of individual adipocytokines, such as leptin, on tumour cell proliferation [34, 35], we observed a significant and dose dependent increase in both oesophageal and colorectal tumour cell proliferation in response to the two cytokines which were significantly higher in visceral adipose tissue, VEGF and IL-6. Neutralization experiments reveal the importance of VEGF in promoting tumour cell proliferation in both oesophageal and colorectal models.

Interestingly, proliferation experiments revealed that vACM from centrally obese cancer patients induced significantly more tumour cell proliferation than their weight matched non-cancer counterparts. This suggests that the presence of cancer in combination with central obesity establishes an environment supportive of further tumour growth and survival, potentially through enhanced production of survival factors or activation of survival signaling pathways in the tumour cells. Potential mechanisms of central obesity and cancer acting additively or synergistically to enhance tumourigenesis warrants further study.

It is important to highlight that while obesity is a major risk factor for the development of these cancers, it is not the only one. Other risk factors include metaplastic conditions such as Barrett's oesophagus and inflammatory bowel disease, along with genetic predisposition, diet and alcohol intake [36, 37]. The pro-angiogenic factors identified in this study, including VEGF, may represent potential mediators responsible for driving tumour progression, thus leading to a poorer overall survival for centrally obese cancer patients. A recent study by Guiu et al. in metastatic colorectal cancer patients, linked excess central adiposity with a poorer outcome following anti-VEGF therapy [38].

In conclusion, analysis of the gene, cytokine and immune profile from visceral and subcutaneous adipose depots in patients with oesophageal and colon cancer highlight that visceral adipose tissue is a rich source of NKT and CD8$^+$ T cells and VEGF. Further work is necessary to link the altered biological milieu caused by central obesity with clinical parameters in cancer patients, including the tumour microenvironment. Increased circulating VEGF associated with central obesity and its key role in inducing tumour cell proliferation, suggests a potentially relevant target for therapeutic intervention in obese cancer patients.

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Figure Legends:

Figure 1. Increased VEGF, IL-6 and leptin gene expression in visceral adipose tissue relative to subcutaneous adipose tissue. Real-time PCR was carried out using specific primer probe sets for IL-8, VEGF, TNF-α, IL-6, adiponectin and leptin. Results show gene fold expression in visceral adipose tissue relative to matched subcutaneous adipose tissue. Results show mean ± s.e.m for n=25 patients. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ by student's t test, comparing gene expression in visceral adipose tissue compared with subcutaneous adipose tissue.

Figure 2. Adipocytokine screen of serum, vACM and sACM. Protein levels of A) IL-8, VEGF, TNF-α and B) IL-6, adiponectin and leptin were determined by ELISA. Significantly higher levels of VEGF and IL-6 were observed in vACM compared with sACM (n=45). Data is expressed as mean ± s.e.m. * $p<0.05$, ***$p<0.001$ by student's t test.

Figure 3: Immunophenotyping of peripheral blood, subcutaneous and visceral adipose tissue. Flow cytometric analysis of the SVF from collagenase digested adipose tissue and peripheral blood. The frequency of mDC, pDC, monocytes, NK, NKT, B cells, CD4+ and CD8+ T cells was assessed. 10,000 events were acquired in the lymphocyte or monocyte gates. Dead cells were identified by propidium iodide staining and quadrant placement was determined using isotype controls. Data are expressed as means ± s.e.m. (n=35). * $p<0.05$ by student's t test.

Figure 4. Cytokine levels in serum and vACM from non-obese and centrally obese patients.

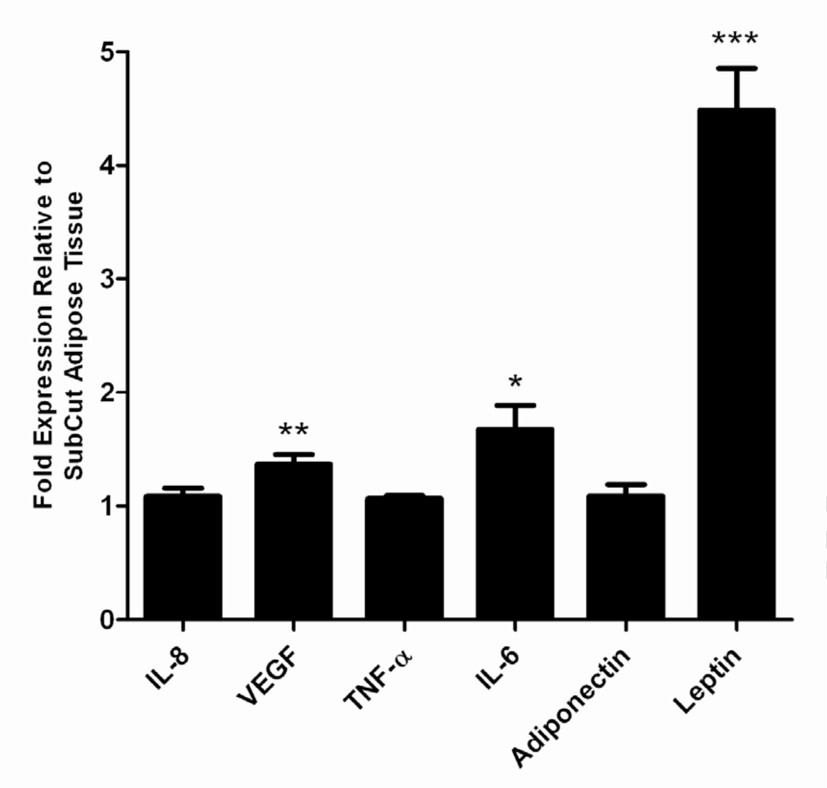
A) Significantly higher levels of VEGF and leptin, and significantly lower levels of adiponectin were observed in the serum of centrally obese patients (n=27) compared with normal weight patients (n=18) assessed by ELISA. B) VEGF in vACM was significantly higher in centrally obese patients compared with non-obese patients. In addition, adiponectin was significantly lower in vACM from centrally obese patients compared to the non-obese patients. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ by student's t test.

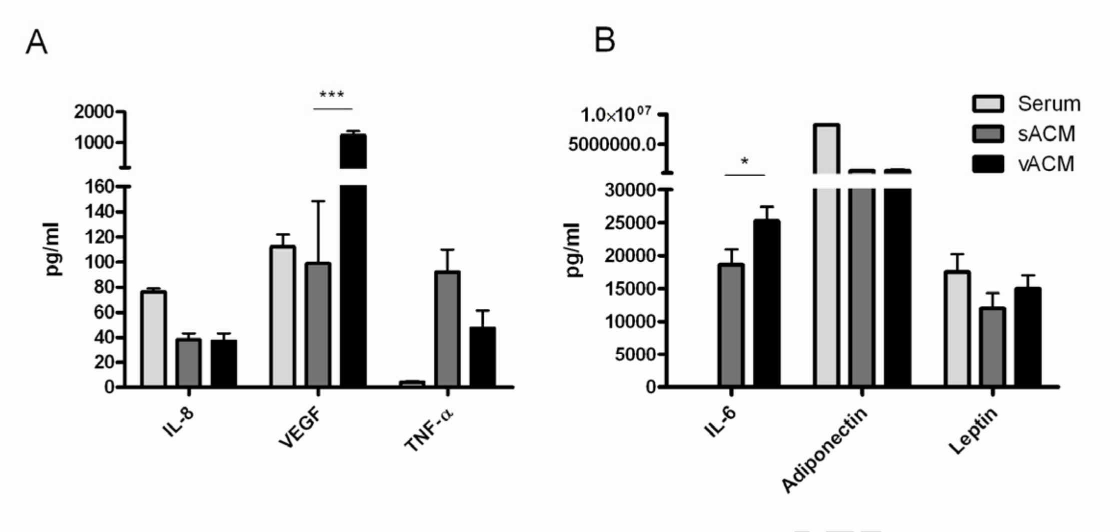
Figure 5: vACM from centrally obese patients significantly increases proliferation of oesophageal and colorectal cancer cells. Oesophageal (OE33) and colorectal (HCT-15) cells were cultured with vACM or sACM from matched patients for 24 hours (n=25). Cell proliferation was assessed by BrdU incorporation assay. Data are expressed as means ± s.e.m. * p<0.05 by student's t test, ns: non-significant.

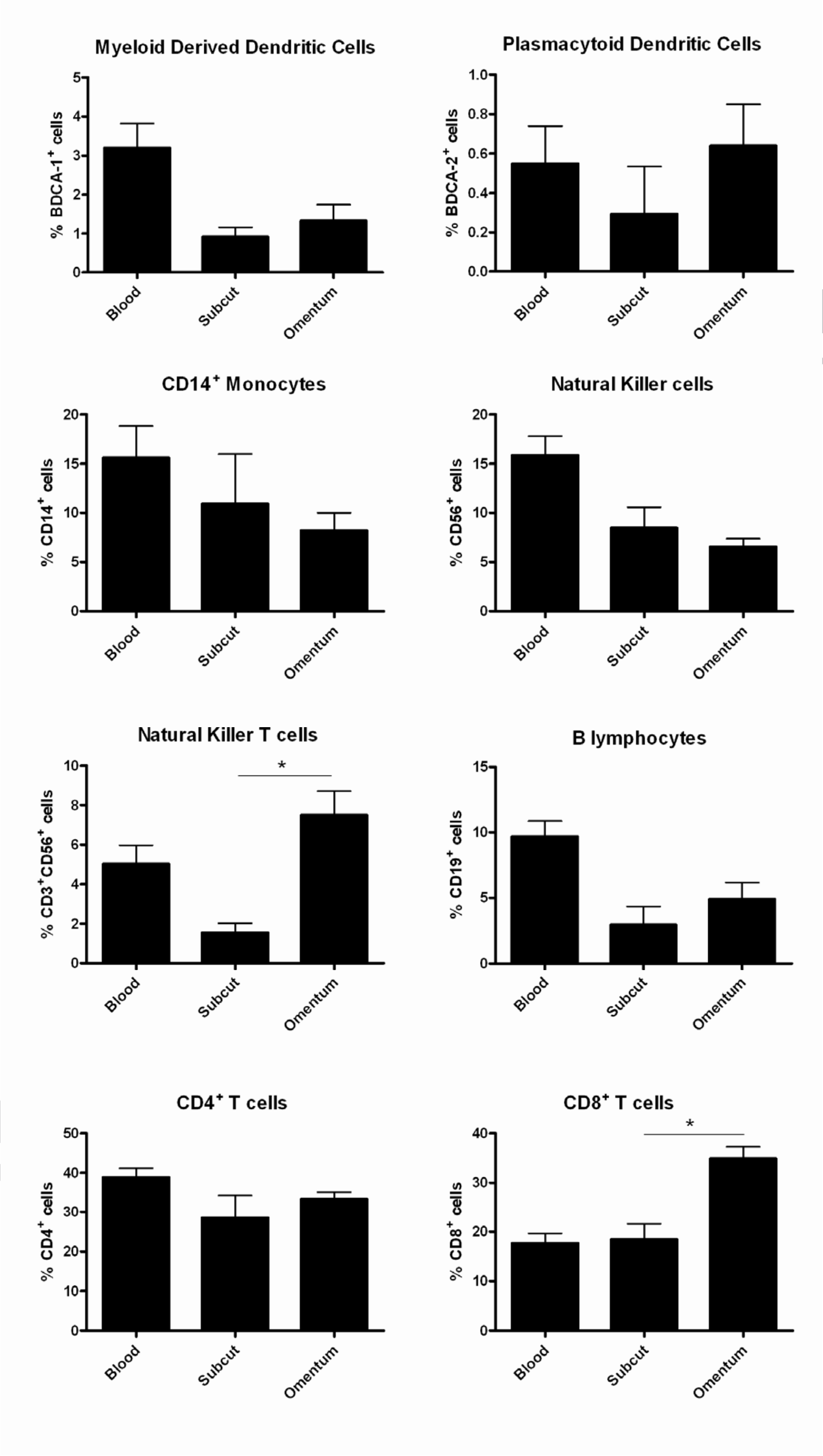
Figure 6: OE33 and HCT-15 show a dose dependent increase in proliferation in response to recombinant IL-6 and VEGF, while neutralizing VEGF significantly reduces proliferation. A) OE33 and HCT-15 were treated for 24 hours with varying concentrations of human recombinant IL-6 and VEGF. Cell proliferation was assessed by BrdU incorporation assay. B) VEGF and IL-6 in vACM from centrally obese patients was removed using specific neutralizing antibodies (NAb) for 24 hours. Cell proliferation was assessed by BrdU incorporation assay. Tumour cells were also treated with M199 media alone as a control, with the baseline set at 100%. Data are expressed as means ± s.e.m. * p<0.05 by student's t test, compared with untreated control, ns: non-significant.

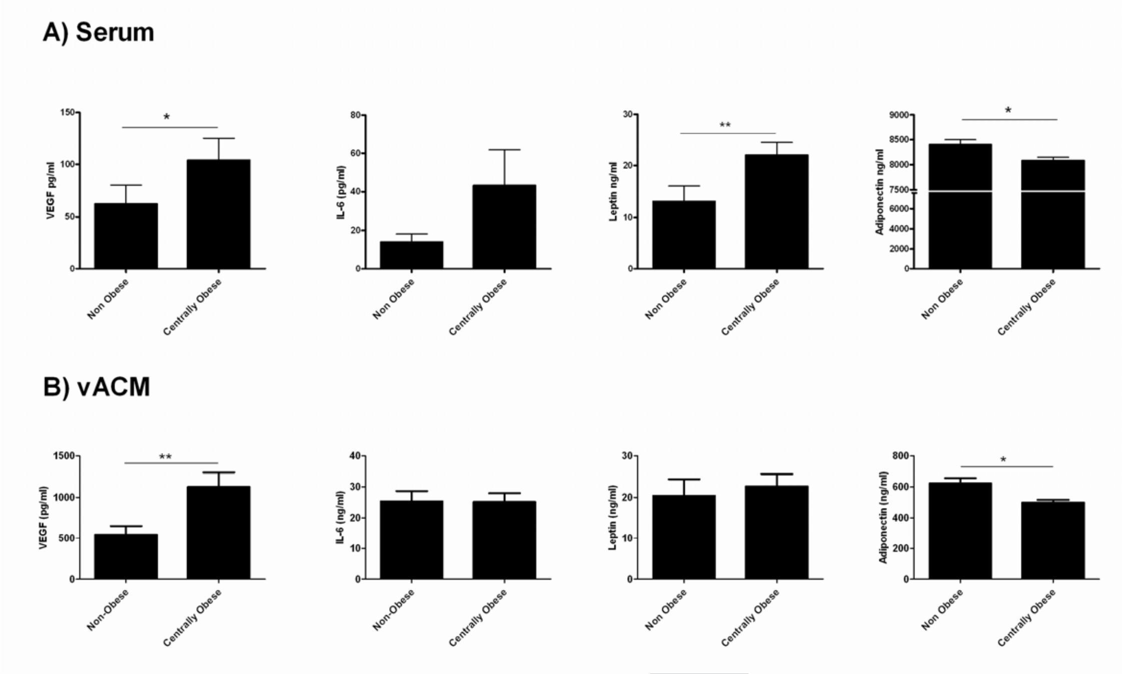
Figure 7: vACM from centrally obese cancer patients induces significantly more tumour cell proliferation than vACM from centrally obese patients without cancer. OE33 and HCT-15 cells were treated with vACM from non-obese non-cancer patients (n=5), obese non-cancer patients (n=6), non-obese cancer patients (n=8) and centrally obese cancer patients (n=10) for 24 hours. Cell proliferation was assessed by BrdU incorporation assay. Tumour cells were also treated with M199 media alone as a control, with baseline proliferation set at 100%. Data are expressed as means ± s.e.m. * p<0.05 and ***p<0.001 by student's t test, ns: non-significant.

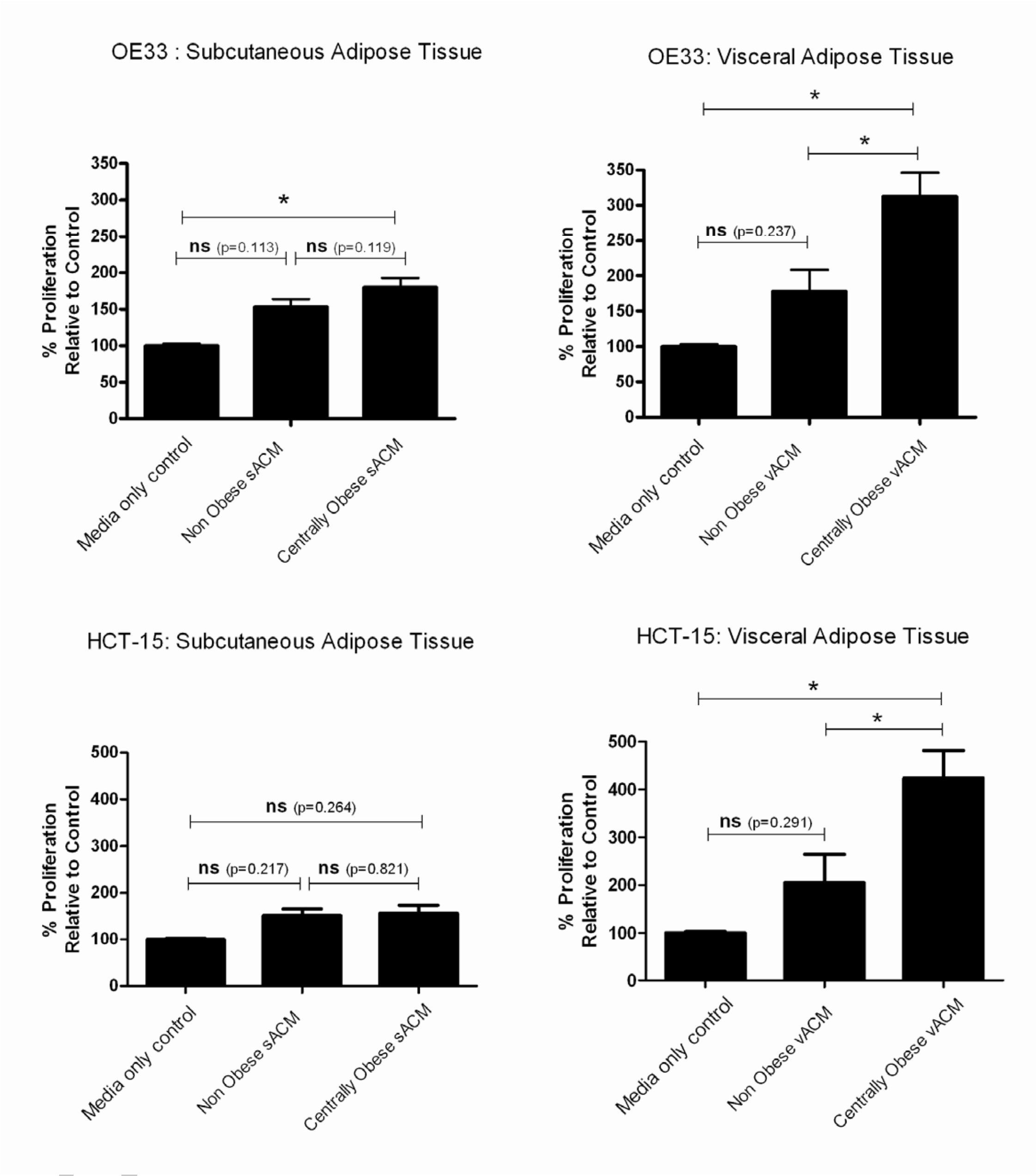
Visceral fat has significantly higher VEGF, $CD8^+$ and NK T cells than subcutaneous fat.
VEGF levels in serum and vACM is significantly higher in viscerally obese patients.
vACM from viscerally obese patients induces significant tumour cell proliferation.
Neutralizing VEGF but not IL-6 in vACM significantly reduces tumour cell proliferation.
VEGF may represent a potential therapeutic target in obese cancer patients.

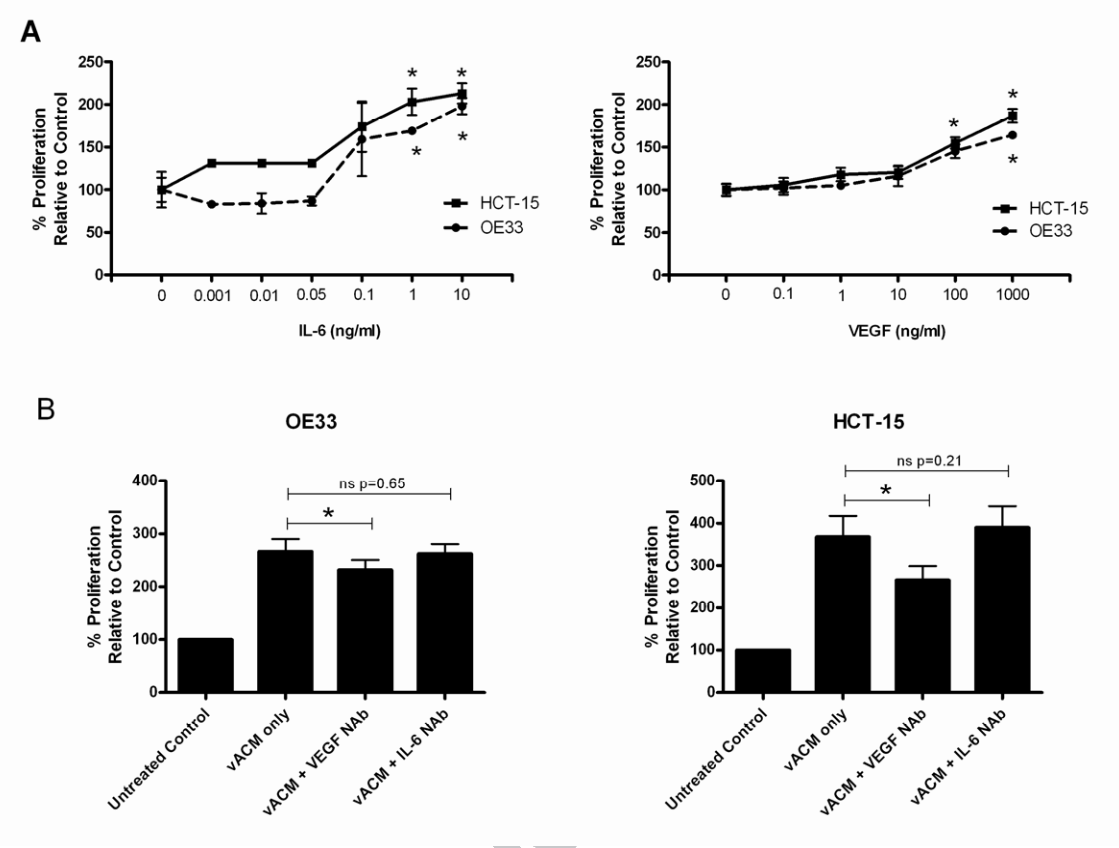

Fold Expression Relative to SubCut Adipose Tissue
0
1
2
3
4
5
**
*
***
IL-8
VEGF
TNF-α
IL-6
Adiponectin
Leptin

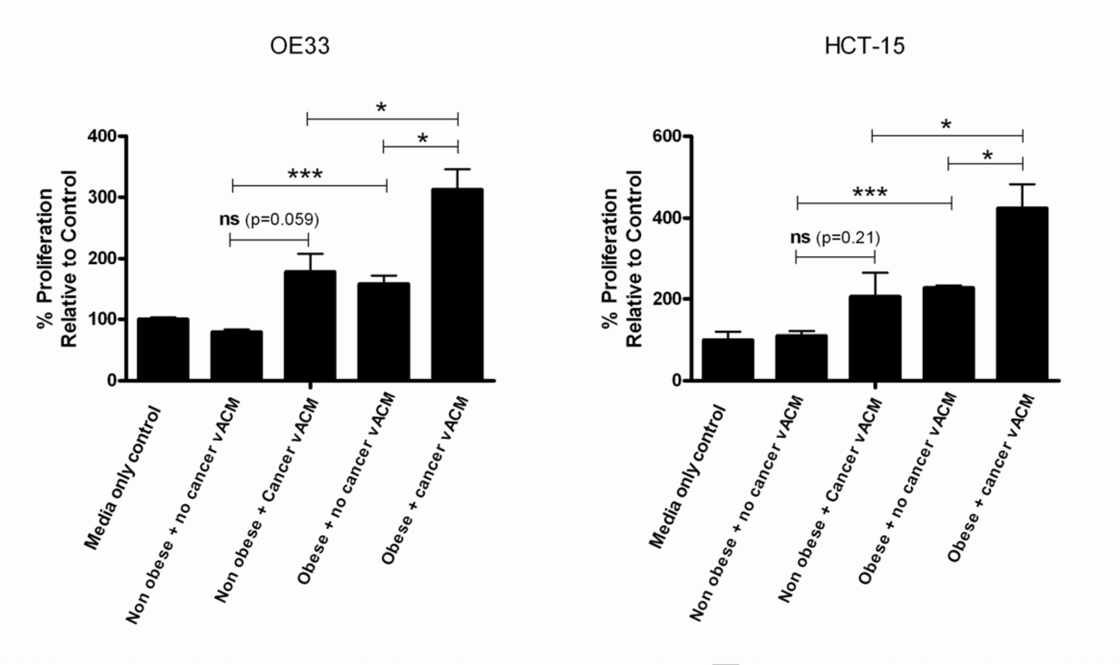

A
B
Serum
sACM
vACM
pg/ml
2000
1000
160
140
120
100
80
60
40
20
0
***
IL-8
VEGF
TNF-α
1.0×10 07
5000000.0
30000
25000
20000
15000
10000
5000
0
*
IL-6
Adiponectin
Leptin


Myeloid Derived Dendritic Cells
% BDCA-1+ cells
0
1
2
3
4
5
Blood
Subcut
Omentum
Plasmacytoid Dendritic Cells
% BDCA-2+ cells
0.0
0.2
0.4
0.6
0.8
1.0
Blood
Subcut
Omentum
CD14+ Monocytes
% CD14+ cells
0
5
10
15
20
Blood
Subcut
Omentum
Natural Killer cells
% CD56+ cells
0
5
10
15
20
Blood
Subcut
Omentum
Natural Killer T cells
% CD3+CD56+ cells
0
2
4
6
8
10
*
Blood
Subcut
Omentum
B lymphocytes
% CD19+ cells
0
5
10
15
Blood
Subcut
Omentum
CD4+ T cells
% CD4+ cells
0
10
20
30
40
50
Blood
Subcut
Omentum
CD8+ T cells
% CD8+ cells
0
10
20
30
40
*
Blood
Subcut
Omentum


A) Serum
VEGF pg/ml
0
50
100
150
*
Non Obese
Centrally Obese
IL-6 (pg/ml)
0
20
40
60
80
Non Obese
Centrally Obese
Leptin ng/ml
0
10
20
30
**
Non Obese
Centrally Obese
Adiponectin ng/ml
0
2000
4000
6000
7500
8000
8500
9000
*
Non Obese
Centrally Obese
B) vACM
VEGF (pg/ml)
0
500
1000
1500
**
Non-Obese
Centrally Obese
IL-6 (ng/ml)
0
10
20
30
40
Non-Obese
Centrally Obese
Leptin (ng/ml)
0
10
20
30
Non Obese
Centrally Obese
Adiponectin (ng/ml)
0
200
400
600
800
*
Non Obese
Centrally Obese


OE33 : Subcutaneous Adipose Tissue
% Proliferation Relative to Control
350
300
250
200
150
100
50
0
*
ns (p=0.113)
ns (p=0.119)
Media only control
Non Obese sACM
Centrally Obese sACM
OE33: Visceral Adipose Tissue
% Proliferation Relative to Control
350
300
250
200
150
100
50
0
*
*
ns (p=0.237)
Media only control
Non Obese vACM
Centrally Obese vACM
HCT-15: Subcutaneous Adipose Tissue
% Proliferation Relative to Control
500
400
300
200
100
0
ns (p=0.264)
ns (p=0.217)
ns (p=0.821)
Media only control
Non Obese sACM
Centrally Obese sACM
HCT-15: Visceral Adipose Tissue
% Proliferation Relative to Control
500
400
300
200
100
0
*
*
ns (p=0.291)
Media only control
Non Obese vACM
Centrally Obese vACM


A
% Proliferation Relative to Control
250
200
150
100
50
0
0
0.001
0.01
0.05
0.1
1
10
IL-6 (ng/ml)
HCT-15
OE33
*
% Proliferation Relative to Control
0
0.1
1
10
100
1000
VEGF (ng/ml)
HCT-15
OE33
*
B
OE33
% Proliferation Relative to Control
400
300
200
100
0
ns p=0.65
*
Untreated Control
vACM only
vACM + VEGF NAb
vACM + IL-6 NAb
HCT-15
500
400
300
200
100
0
ns p=0.21
*
Untreated Control
vACM only
vACM + VEGF NAb
vACM + IL-6 NAb


OE33
HCT-15
% Proliferation Relative to Control
0
100
200
300
400
600
ns (p=0.059)
ns (p=0.21)
***
*
*
Media only control
Non obese + no cancer vACM
Non obese + Cancer vACM
Obese + no cancer vACM
Obese + cancer vACM

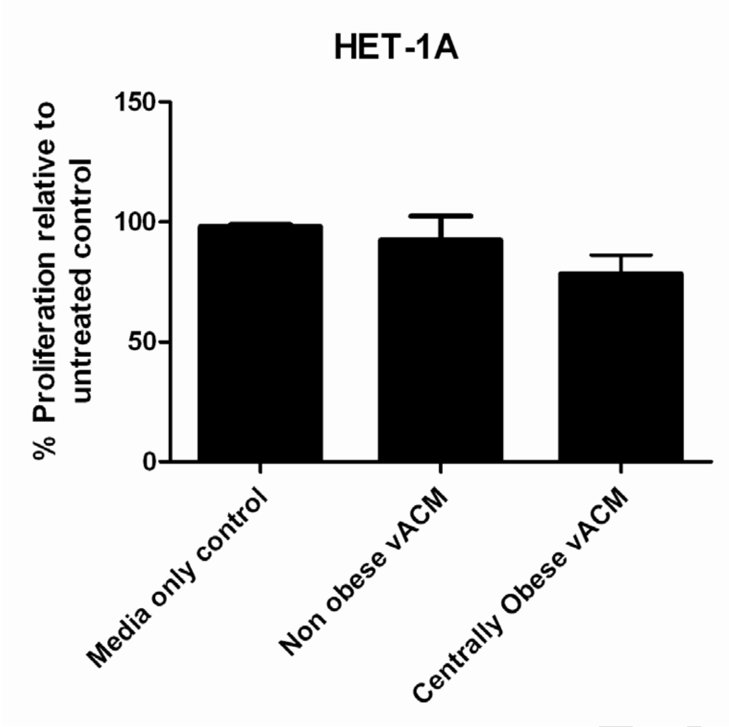

HET-1A
% Proliferation relative to untreated control
150
100
50
0
Media only control
Non obese vACM
Centrally Obese vACM

Table 1: Patients Demographics

n=281		n	%
Age (years)	Mean + SEM	66.4 +/- 0.6	
	Range	31 - 96	
Gender	Male	185	66%
Cancer type	Oesophageal Adenocarcinoma	119	42.4%
	Oesophageal Squamous Cell Carcinoma	28	9.9%
	Gastric cancer	21	7.5%
	Colorectal Adenocarcinoma	113	40.2%
[a]**Body Mass Index**	Mean + SEM	25.3+/-0.24	
	Range	15.43-41.02	
	Underweight (< 19.99)	18	7.3%
	Normal (20-24.99)	90	36.4%
	Overweight (25-29.99)	99	40.1%
	Obese (> 30)	40	16.2%
[b]**Central Waist Circumference (cm)**	Mean + SEM	92.45 +/- 0.72	
	Range	61-130	
	Normal	114	43.7%
	Central Obesity (>80cm female, >94cm male)	147	56.3%
[c]**CT Visceral Fat Area (cm²)**	Mean +/- SEM	142.97 +/- 8.2	
	Range	5.8 - 338.5	
	Normal	72	44.7%
	CT Viscerally Obese (VFA >130cm²)	89	55.3%
CT Subcutaneous Fat Area (cm²)	Mean +/- SEM (cm²)	176.0 +/- 7.88	
	Range	5.4 - 479.7	
[d]**Metabolic Syndrome Status**	Meets criteria (IDF 2006)	89	36.6%

[a] BMI was available for 247 patients only

[b] Waist circumference was available for 261 patients only

[c] CT scans were available for 161 patients only

[d] Metabolic syndrome status available for 243 patients only

IDF: International Diabetes Federation Definition 2006