Endothelial Progenitor Cells are Increased in Patients with Advanced Pancreatic Cancer Treated with Gemcitabine and Intravenous Omega 3 Compared with Controls

John Isherwood (John.d.isherwood@outlook.com)
University of Leicester
Ali Arshad
University Hospital Southampton NHS Foundation Trust
Wen Chung
University Hospitals of Leicester NHS Trust
Franscois Runau
University Hospitals of Leicester NHS Trust
Jill Cooke
University Hospitals of Leicester NHS Trust
Cristina Pollard
University Hospitals of Leicester NHS Trust
Lynne Howells
University of Leicester
Jenny Fishwick
University of Leicester
John Thompson
University of Leicester
Matthew Metcalfe
University Hospitals of Leicester NHS Trust
William Steward
University of Leicester
Ashley Dennison
University Hospitals of Leicester NHS Trust

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Abstract

Introduction Pancreatic adenocarcinoma (PAC) is a devastating disease. Endothelial progenitor cells (EPCs) are important mediators in PAC. Omega 3 fatty acids (ω-3FAs) have been shown to have anti-inflammatory properties.

Methods This was a single centre study investigating intravenous ω-3FAs and gemcitabine chemotherapy versus gemcitabine therapy alone in patients with APC. The primary outcome measure was the level of EPCs with the secondary outcome measure being the relationship between the EPC levels, progression free survival and overall survival.

Results Over the treatment period $CD45^-$, $CD31^+$, $CD133^+$ EPCs and $CD45^-$, $CD31^+$, $CD34^+$ EPCs significantly increased (P=0.042 and P=0.0001), whilst no change was observed in control EPCs (P=0.705). There was a non-significant downward trend in control EPCs (P=0.930, 95%). There was a significant difference between the trial and control patients over time on logistic regression analysis (P=0.0001). There was a significant increase in $CD45^-$, $CD34^+$, $CD31^+$ and $CD133^+$ EPCs trial EPCs over treatment (P=0.007). There was no significant increase seen in control EPCs (P=0.358, 95%). There was a significant difference between the trial and control patients over treatment on logistic regression analysis (P=0.0001). There was a significant correlation in the trial patients between progression free survival benefit and an increase in EPCs.

Conclusion Intravenous ω-3FAs with gemcitabine chemotherapy in APC results in a significantly increased level of EPCs. There appears to be a survival benefit in patients with an increase in EPCs which may be the result of a reduction in pro-inflammatory mediators.

Introduction

Pancreatic adenocarcinoma (PAC) is a devastating diagnosis with the majority of patients presenting with advanced pancreatic cancer (APC). The incidence of the disease approximates to the mortality, with around 7,500 to 8,000 new cases and deaths each year in the UK, making it the 5th most common cause of cancer death(1). For those diagnosed with unresectable cancer the life expectancy is approximately 8 months(2, 3). Pancreatic adenocarcinoma almost ubiquitously metastasises, predominantly to the liver, lungs and abdomen and invades important surrounding anatomical structures at an early stage(4). Of all presenting pancreatic adenocarcinomas cancers, approximately 20% will be operable with curative intent with the remaining majority receiving palliative treatment(5).

EPCs

Vasculogenesis is the process by which blood vessels are formed *de novo* and angiogenesis is the expansion and remodelling of the existing blood vessel network(6). The process of angiogenesis is regulated by multiple pro- and anti- angiogenic mediators and growth factors. The imbalance of these anti- and pro- angiogenic factors activates an “angiogenic switch”(7), and involves a myriad participating cells. Tumours can form vessels by “hijacking” neighbouring pre-existing vessels(8) but there is increasing evidence that vasculogenesis, through which bone marrow derived hematopoietic stem cells and endothelial progenitors home into to tumour sites(9), plays an important role. Cancer progression requires new vessel formation to deliver oxygen, nutrients and growth factors and tumours are therefore key promoters of vasculogenesis and angiogenesis. Tumours such as PAC have a hypoxic environment and circulating endothelial progenitor cells (EPCs) are mobilised in response to the tissue hypoxia which ensures the promotion of angiogenesis. A number of studies have shown that EPCs have the ability to form colonies *in vitro*, highlighting their role in angiogenesis(10), the maintenance of existing vascular structures(11), and tumour vasculogenesis(12) and as a consequence, changes in these cell numbers could potentially be advantageous in a clinical setting.

Omega 3 Fatty Acids

ω-3FAs have been shown to have anti-inflammatory properties and there is abundant research demonstrating the benefit of ω-3FAs in PAC, from cell culture and animal models through to human clinical trials. *In vitro* ω-3FAs have been shown to inhibit the growth of human pancreatic cancer, augment the effect of gemcitabine, induce apoptosis, and inhibit the proliferation and invasion of PAC(13, 14).

The incorporation of ω-3FAs into the cell membrane alters its composition and they play an important role in membrane protein function and intracellular fatty acid receptors(15), maintaining membrane fluidity(16), influencing lipid raft formation(17) and crucially are metabolised to secondary messengers and metabolites(18) (Fig. 1). Intravenous ω-3FAs may also change the EPC profile seen in PAC conferring clinical benefit and indicating a potential area for future therapeutic strategies.

Figure 1. Eicosanoids derived from ω-3 FAs (EPA and DHA) and ω-6FAs (AA). They are metabolised by cyclooxygenase and lipoxygenase. The anti-inflammatory eicosanoids produced by ω-3FAs include the 3 series prostaglandins and thromboxanes and the 5 series leukotrienes. The pro inflammatory eicosanoids produced by ω-6FAs include the 2 series prostaglandins and thromboxanes and the 4 series leukotrienes. Increasing the amount of ω-3FAs improves the ω-3 FAs:ω-6FAs ratio and reduces the amount of ω-6FAs, particularly AA, present in the cell membrane and therefore the amount available for downstream pro inflammatory eicosanoid production. All these secondary mediators have either a general suppressive or enhancing effect on growth factors and cells in the inflammatory microenvironment.

Methods

Twenty-seven patients were recruited, eighteen in a non-randomised manner as part of a phase two, single arm, and single-centre study of gemcitabine plus intravenous ω-3FAs in patients with chemotherapy-naïve advanced pancreatic cancer. This trial was registered with cinicaltrials.gov: NCT01019382. Nine subsequent control patients who had received standard gemcitabine monotherapy were also recruited in a non-randomised manner. The local Ethics Committee and the Medicines and Healthcare Products Regulatory Agency (MHRA) approved both studies. All patients had a histological diagnosis of APC. All patients were discussed at the local multi-disciplinary team meeting and were assessed by an oncologist and considered suitable to receive gemcitabine chemotherapy. All patients were assessed against trial protocol inclusion/exclusion criteria.

Treatment protocol

Patients received a standard dose of gemcitabine (1000 mg/m^2) administered as a thirty minute infusion once weekly for three weeks, followed by a one week break from treatment up to a maximum of six months. Immediately following each administration of gemcitabine patients received up to 500 mL of a lipid emulsion intravenously (Lipidem, BBraun) containing 10 g ω-3 fatty acids (0.5-1 g ALA and 4.3–8.6 g EPA/DHA) over four hours and all patients received at least 250 mL of Lipidem™. Control patients received gemcitabine treatment alone at a standard dose prescribed weekly by an oncologist with dose adjustments as per standard clinical practice. Patients were included in the study until progression of disease, death, serious adverse events necessitating withdrawal or patients withdrawing from treatment. A CT scan was performed at baseline, at any stage if disease progression was suspected, or every eight weeks. Patients continued in the trial as long as their tumour did not demonstrate progression as defined by the modified Response Evaluation Criteria in Solid Tumours (RECIST) criteria which was assessed by an independent radiologist. Blood samples were obtained at each treatment time point and kept on ice prior awaiting sample processing which occurred immediately following collection.

PMBC separation, storage and preparation

Blood samples consisting of 19.6 mL of whole blood were collected in 4 × 4.9 mL Ethylenediaminetetraacetic acid (EDTA) bottles from each patient at each treatment point following the completion of the treatment. Peripheral blood mononuclear cells (PBMCs) were isolated using standard protocols with a density gradient separation solution (Ficoll-Paque™ PREMIUM 1.084) and stored in an -80 °C freezer. Samples were snap-thawed at 37 °C in a temperature controlled water bath. The samples were

washed and isolated and the cell number and percentage of viable cells was then determined prior to antibody staining using the cell counting protocol.

Sample staining and Flow cytometry analysis

PBMCs were analysed for EPCs. EPCs with three antibody phenotypes were analysed: 1. $CD45^-$, $CD31^+$ and $CD133^+$, 2. $CD45^-$, $CD31^+$and $CD34^+$ and 3. $CD45^-$, $CD31^+$, $CD133^+$ and $CD34^+$. Antibodies utilised to identify EPCs included CD31 FITC Mouse Anti-Human. BD Pharmingen®, CD45 Pacific Blue Mouse Anti-Human. BD Pharmingen® (Berkshire, UK), CD34 PE Mouse Anti-Human. BD Pharmingen® and CD133 APC. Miltenyi Biotec® (Surrey, UK). Samples were analysed for unstained cells, each individual antibody and a combination stain. Cells were processed using standard protocols. Samples were analysed with the FACS Aria II flow cytometer (Becton Dickinson, BD Biocsiences, San Jose, USA). Each sample was analysed for the unstained cells, each individual antibody evaluation and the overall combination analysis (Fig. 2).

Figure 2. FACS schematic scatter plots of EPCs with a quadruple stain for $CD45^-$, $CD31^+$, $CD133^+$ and $CD34^+$ antibodies. The top left plot shows side scatter (SSC) versus forward scatter (FSC). A mean of 374,796 cells in the target population (P1) were analysed over the one hundred and thirty-four samples investigated. Three populations of EPCs were analysed. EPCs with a $CD45^-$, $CD31^+$, $CD133^+$ and $CD34^+$ phenotype are seen in Q2.2 ($CD133^+$ (APC) and $CD34^+$ (PE)), gated off P6 ($CD31^+$ (FITC) & $CD45^-$ (R/B220 pacific blue). EPCs with a $CD45^-$, $CD31^+$and $CD34^+$ phenotype are seen in Q2.1 ($CD34^+$ (PE) and $CD31^+$ (FITC)), gated off P3 ($CD45^-$ (R/B220 pacific blue). EPCs with a $CD45^-$, $CD31^+$and $CD133^+$ phenotype are seen in Q2 (CD31+ (FITC) and CD133+ (APC)), gated P3 ($CD45^-$ (R/B220 pacific blue).

Statistical analysis

Overall and progression free survival data was analysed with Kaplan-Meir curves and a log-rank (Mantel-Cox) test. Clinical outcomes were correlated with changes in mediators and survival curves analysed with a log-rank (Mantel-Cox) test. Changes in cells over the trial in both trial and control patients and between groups were analysed with logistic regression analysis using STATA software. A mixed effects logistic regression model was utilised that allowed for random variation and missed time points. These statistical models were chosen as the clinical trial data varied in length depending on individual treatment, in addition to missing time points resulting from the unavoidable factors, both patient and investigator related, in a clinical trial.

EPC measurement

EPCs are rare cells and make up between 0.01% and 0.001% of mononuclear cells in normal peripheral blood(19). The number of circulating $CD34^+$ cells is around 50–100 per million white blood cells (0.005 – 0.001%), equal to about 350–700 cells per mL(20), and there are some report that suggest co-expression of CD133 increases the specificity for EPCs as it is not expressed on mature endothelial cells(20, 21). This co-expression of CD34 and CD133 cells in peripheral blood is even lower and makes quantification significantly more difficult. In order to compensate for this, most researcher groups acquire large amounts of cells or events. Strict criteria for the separation of cells, titrating of antibodies and the use of high-end flow cytometry machines and methods are essential. No universal agreement regarding phenotypic identification and lack of methodological consensus compounds the variability in the published literature. As a consequence there is wide variability in reported phenotypic subtypes that include a variety of markers (Table 1). There are also inconsistencies in the reporting of results, with some studies reporting EPCs for a sample volume and EPCs for a defined number of mononuclear cells(22). EPCs in this study were separated, processed, analysed and identified according to literature standards. EPCs are reported as a percentage of the parent population and three EPC phenotypes were examined.

Table 1
Antibody phenotypes and characteristics from select studies.

Study	Pathology	Subjects	Antibody					
			CD45-	CD31+	CD133+	CD34+	CD146-	VEGFR2
Vizio et al (2010)[8]	PAC	Human	Y	Y	Y	Y		
Sakamori et al (2012)[54]	NSCLC	Human	Y	Y	Y	Y		
Morita et al (2011)[55]	NSCLC	Human	Y	Y	Y	Y		
Steurer et al (2008)[56]	NSCLC	Human	Y	Y	Y	Y		
Roodhard et al (2010)[57]	Various*	Human	Y	Y	Y			
Li et al (2011)[58]	PAC	Mouse			Y	Y	Y	
Staringer et al (2011)[59]	PAC	Human	Y	Y			Y	
Kuo et al (2012)	Breast	Human	Y	Y	Y		Y	
Lin et al (2013)[60]	Rectal cancer	Human	Y	Y	Y			Y
Fuereder et al (2014)[61]	Prostate cancer	Human	Y	Y	Y		Y	
DuBois et al (2012)[62]	Osteosarcoma	Human	Y	Y	Y		Y	
Corsini et al (2012)[63]	Glioma	Human	Y		Y	Y		
Kim et al (2013)[64]	Gynaecological cancers	Human	Y		Y	Y		Y
Bhatt et al (2011)[65]	Renal cell carcinoma	Human	Y		Y	Y	Y	
Ha et al (2013)[66]	Gastric cancer	Human			Y	Y		
Marlicz et al (2016)[67]	Colorectal cancer	Human	Y		Y	Y		Y
Starzynske et al (2013)[40]	PAC	Human	Y	Y		Y		Y
Ko et al (2010)[42]	PAC	Human	Y	Y		Y		
Steurer et al (2008)[56]	NSCLC	Human	Y	Y		Y		Y
Shim et al (2015)[68]	Myocardial infarction	Human	Y	Y		Y	Y	
Mancuso et al (2011)	Breast cancer	Human	Y	Y		Y		

Table 1. Demographic characteristics of the study participants and the number of treatment time points and completed cycles per group. A treatment cycle included three treatment points (each week) and a rest week.

Table 1. Antibody phenotypes and characteristics from select studies. There is wide discrepancy in the antibody phenotypes used to identify EPCs. In PAC the antibody phenotype of $CD45^{-}$, $CD31^{+}$, $CD34^{+}$ & $CD133^{+}$ [8] and $CD45^{-}$, $CD31^{+}$ and $CD34^{+}$ [40,42]. However the phenotype of $CD45^{-}$, $CD34^{+}$ & $CD133^{+}$ has been investigated in various other cancers as shown above. NSCLC: Non-small cell lung cancer. VEGFR2: Vascular endothelial growth factor receptor 2. PAC: Pancreatic adenocarcinoma. Various*: Cancers studied include breast, colorectal, ovarian, oesophagus, prostate, head and neck, sarcoma, cervical and others not

described. CD45: A hematopoietic marker also known as the Leukocyte Common Antigen which is present on all human leucocytes, including lymphocytes, monocytes, granulocytes, eosinophils and thymocytes. It is absent from erythrocytes, platelets or mature erythroid cells of bone marrow and non-haemopoietic tissues. CD31: An endothelial cell marker also known as PECAM-1 (Platelet And Endothelial Cell Adhesion Molecule 1), It is implicated in angiogenesis [69], vascular wound healing and transendothelial migration of leukocytes in inflammatory processes. It is widely expressed on endothelial cells as well as platelets, monocytes and granulocytes. CD34: An endothelial cell marker also known as the hematopoietic progenitor cell antigen, it is expressed on hematopoietic progenitor cells, vascular endothelium and some tissue fibroblasts. CD133: An early hematopoietic stem cell marker, CD133 is expressed on circulating endothelial progenitor cells[21,31]. In the hematopoietic system, CD133 expression is restricted to a subset of $CD34^{bright}$ stem and progenitor cells in human foetal liver, bone marrow, cord blood and peripheral blood [70]. CD146: Also known as melanoma cell adhesion molecule or MCAM it belongs to the immunoglobulin superfamily. It is expressed in epithelial cells, activated T cells, endothelial cells and multipotent mesenchymal stromal cells[71]. VEGFR-2: An endothelial marker.

Results

Patient demographics are shown in Table 2. A total of twenty-seven patients were recruited, eighteen trial and nine control patients. EPCs with three antibody phenotypes were analysed. There was a significant increase in $CD45^{-}$, $CD31^{+}$and $CD133^{+}$ EPCs over the treatment period (P = 0.042, Fig. 3). There was no significant change in EPCs in the control group (P = 0.705, 95% CI= -0.023–0.034). Overall there was no significant difference between the trial and control patients over treatment on logistic regression analysis (P = 0.332). There was a significant increase in $CD45^{-}$, $CD31^{+}$ and $CD34^{+}$ EPCs over the treatment period (P = 0.0001, Fig. 4). There was a non-significant downward trend in control EPCs seen (P = 0.930, 95% CI= -0.029–0.027). Overall there was a significant difference between the trial and control patients over time on logistic regression analysis (P = 0.0001). In $CD45^{-}$, $CD34^{+}$, $CD31^{+}$ and $CD133^{+}$ EPCs there was a significant increase in trial EPCs over treatment (P = 0.007, Fig. 5). There was no significant increase seen in control EPCs (P = 0.358, 95% CI= -0.016–0.466). There was a significant difference between the trial and control patients over the treatment period on logistic regression analysis (P = 0.0001).

Table 2
Patient demographics.

Demographics		Trial patients N = 18	Control Patients N = 9
Gender	Male	10	7
	Female	8	2
Age	Median age (range)	70 (59–83)	64 (50–75)
	> 70 years	8	2
	< 70 years	10	8
Ethnicity	White British	16	8
	Asian	2	1
Baseline Weight	Median weight in Kilograms (range)	62.1 (48.6–81)	69.1 (54.2–86)
Stage	Stage 3	7	5
	Stage 4	11	4
Total number of treatment time points (median)		146 (6.5)	38 (3)
Number of patients completed 1 cycle (%)		14 (78%)	5 (63%)
Number of patients completed 2 cycles (%)		11 (61%)	2 (25%)
Number of patients completed 4 cycles (%)		7 (39%)	1 (12.5%)
Number of patients completed 6 cycles (%)		4 (22%)	1 (12.5%)

Table 2. Antibody phenotypes and characteristics from select studies. There is wide discrepancy in the antibody phenotypes used to identify EPCs. In PAC the antibody phenotype of $CD45^-$, $CD31^+$, $CD34^+$ & $CD133^+$(8) and $CD45^-$, $CD31^+$ and $CD34^+$ (40,42). However the phenotype of $CD45^-$, $CD34^+$ & $CD133^+$ has been investigated in various other cancers as shown above. NSCLC: Non-small cell lung cancer. VEGFR2: Vascular endothelial growth factor receptor 2. PAC: Pancreatic adenocarcinoma. Various*: Cancers studied include breast, colorectal, ovarian, oesophagus, prostate, head and neck, sarcoma, cervical and others not described. CD45: A hematopoietic marker also known as the Leukocyte Common Antigen which is present on all human leucocytes, including lymphocytes, monocytes, granulocytes, eosinophils and thymocytes. It is absent from erythrocytes, platelets or mature erythroid cells of bone marrow and non-haemopoietic tissues. CD31: An endothelial cell marker also known as PECAM-1 (Platelet And Endothelial Cell Adhesion Molecule 1), It is implicated in angiogenesis (54), vascular wound healing and transendothelial migration of leukocytes in inflammatory processes. It is widely expressed on endothelial cells as well as platelets, monocytes and granulocytes. CD34: An endothelial cell marker also known as the hematopoietic progenitor cell antigen, it is expressed on hematopoietic progenitor cells, vascular endothelium and some tissue fibroblasts. CD133: An early hematopoietic stem cell marker, CD133 is expressed on circulating endothelial progenitor cells(21,31). In the hematopoietic system, CD133 expression is restricted to a subset of $CD34^{bright}$ stem and progenitor cells in human foetal liver, bone marrow, cord blood and peripheral blood (55). CD146: Also known as melanoma cell adhesion molecule or MCAM it belongs to the immunoglobulin superfamily. It is expressed in epithelial cells, activated T cells, endothelial cells and multipotent mesenchymal stromal cells(56). VEGFR-2: An endothelial marker.

Demographic characteristics of the study participants and the number of treatment time points and completed cycles per group. A treatment cycle included three treatment points (each week) and a rest week.

Figure 3. Output graph showing regression lines for individual patients plotted against time in weeks following mixed effects logistic regression analysis. EPCs levels are shown on the y axis and are a calculated as a percentage of the parent population (log scale), time in weeks is shown on the x axis. Each regression line represents a patient. One hundred and three time points in eighteen patients were analysed. There was a significant increase in EPCs over treatment (P = 0.042, 95% CI = 0.0008–0.046).

Figure 4. Output graph showing regression lines for individual patients plotted against time in weeks following mixed effects logistic regression analysis. EPCs levels are shown on the y axis and are a calculated as a percentage of the parent population (log scale), time in weeks is shown on the x axis. Each regression line represents a patient. One hundred and two time points in eighteen patients were analysed. There was a significant overall increasing trend in EPCs over time (P = 0.0001, 95% CI = 0.132–0.385).

Figure 5. Output graph showing regression lines for individual patients plotted against time in weeks following mixed effects logistic regression analysis. EPCs levels are shown on the y axis and are a calculated as a percentage of the parent population (log scale), time in weeks is shown on the x axis. Each regression line represents a patient. Twenty-seven samples in nine patients were analysed. EPCs are calculated as a percentage of the parent population and plotted on a log scale. One hundred and three time points in eighteen patients were analysed. There is a significant increase in EPCs over time (P = 0.007, 95% CI = 0.006–0.377).

Survival analysis

EPCs in both trial and control patients were divided into two groups (low and high change groups) around the median percentage change in EPCs between baseline and end point. Overall survival (OS) and progression free survival (PFS) of trial and control patients were compared in the low and high change groups. Patient T10 was excluded from this analysis as the patient became operable. Patients C5, C7, C9, T7, T12 and T14 were excluded from the analysis as they had no sample following a baseline measurement.

In $CD45^-$, $CD31^+$and $CD133^+$ EPCs there was a significant PFS benefit in trial patients demonstrating a large change in EPCs versus control patients (P = 0.0023). There was also a significant OS benefit seen in trial patients with a large change in EPCs compared with control patients (P = 0.019). In $CD45^-$, $CD31^+$ and CD34 EPCs there was no significant difference in PFS in patients. There was a minimal change in EPCs in trial versus control patients (P = 0.42). There was a significant PFS benefit in patients with a large change in EPCs in trial versus control patients (P = 0.012). There was no difference in OS in patients irrespective of the level of change of EPCs. In $CD45^-$, $CD34^+$, $CD31^+$ and $CD133^+$ EPCs there was a significant PFS benefit in trial patients with a small change in EPCs versus controls (P = 0.0008). In addition, there was a significant PFS benefit in trial patients with a large change in EPCs in trial versus control patients (P = 0.012). There was no difference in OS in patients irrespective of the degree of change in EPCs levels in trial versus control patients.

Discussion

This study aimed to assess the effect of administering intravenous ω-3FAs in combination with gemcitabine chemotherapy on EPCs in patients with APC compared to gemcitabine monotherapy alone. Previous published data have demonstrated that intravenous administration of ω-3FAs is well tolerated(23), results in rapid uptake into cell membranes and improves patient's quality of life(24–28) in advanced cancers. This study has some limitations. Firstly the trial and control groups are unmatched, with eighteen in the trial cohort and nine in the control cohort. Recruitment was limited to nine control patients as a result of the standard chemotherapy regime being changed following the introduction of nab-paclitaxel as a chemotherapeutic agent administered in addition to gemcitabine(29). This study compared treatment in two cohorts with patients recruited in succession as they presented and randomisation of patients and treatment would have reduced selection bias.

In this study we investigated the addition of Lipidem to standard gemcitabine chemotherapy. Lipidem contains 20 g ω-3FAs and 8.6–17.2 g of EPA and DHA in 1000 mL. Ideally a more refined investigational product that contained a purer form of ω-3FAs in a lower total volume would have been chosen. Lipidem was administered following the gemcitabine treatment as per standard protocol on days 1, 8 and 15, which was followed by a rest week. This was as a result of the Ethics Committee review that believed it was unacceptable to inconvenience patients by deviating from their standard treatment protocols with additional visits to the oncology department. Patients would ideally receive intravenous ω-3FAs every week in order to maintain levels but

altering the treatment course or administering high dose oral supplementation that would support intravenous administration is an alternative approach.

This study is the first to analyse EPCs at multiple treatment points in patients receiving intravenous ω-3FAs. The majority of other published studies only analyse baseline mediator levels and at one, two or three treatment points. There is general variability in EPC levels seen at each treatment point and multiple time point analysis is required to provide an accurate assessment of levels over a treatment period. This study provides an important and robust analysis of EPC changes which occur over a whole treatment period.

EPCs

In 1997 Asahara and colleagues isolated $CD34^+$ hematopoietic cells in human peripheral blood (20, 30) which stimulated further investigation of endothelial progenitor cells. However, defining the exact phenotypic identification proved challenging (Table 2). EPCs are a subtype of stem cells with a high proliferative potential that are capable of differentiating into mature endothelial cells and contributing to neovascularization(6, 31). EPCs have the ability to migrate, colonise, proliferate and ultimately, differentiate into endothelial lineage cells(32). These progenitor cells are in a state of transformation and can be considered part of a development continuum. EPC recruitment and mobilisation have been shown to correlate with increased levels of angiogenic growth factors including vascular endothelial growth factor (VEGF)(33). VEGF is responsible for the proliferation, differentiation and chemotaxis of EPCs(32, 34) and is released from tumour cells, macrophages and platelets(35). Other mediators and growth factors stimulating EPC recruitment include fibroblast growth factor (FGF), angiopoietin-1, stromal derived factor-1 and placental growth factor(36–38).

The cancer stem cell hypothesis proposes a hierarchical organisation of tumours, in which a subpopulation of stem cell-like cells sustains tumour growth, metastasis and resistance to therapy(39). With recognition of the importance of these cells research into their specific elimination and manipulation becomes clinically relevant. There are myriad stem and progenitor cells involved in the tumorigenesis of pancreatic cancer including cancer stem cells, haematopoietic stem cells, very small embryonic/epiblast-like stem cells and endothelial stem/progenitor cells(40). The possible mechanism for the increase in EPCs in cancer may include the generalised activation of the endothelium, localised endothelial damage or an elevation in stimulating growth factors and mediators mobilising precursor cells(8). In addition, pancreatic cancer is notoriously hypoxic with an inflammatory stromal cuff that results in tumour release of mediators that result in EPC recruitment. However the mobilisation, recruitment and incorporation of EPCs into tumours is a complex and multi factor process that involves the participation of numerous mediators and cells in the tumour microenvironment(9) and the complete mechanisms are yet to be fully understood.

EPCs have been shown to be elevated in a number of different cancers (Table 2), and there is a recognised increase in EPC number in the peripheral blood of patients with increased tumour burden(41). Vizio *et al*(8) demonstrated that levels of EPCs were between 10 and 26 times higher in APC patients compared to healthy controls(8). They further demonstrated that increased EPC levels were significantly correlated with an increased TNM stage, a deteriorating prognosis and a poorer overall survival(8). In addition Ko *et al*(42) also demonstrated that baseline EPC concentration was inversely associated with overall survival in patients treated with bevacizumab plus erlotinib for gemcitabine refractory APC. There is a paucity of studies into the effect of chemotherapy regimes on EPCs in APC although there have been studies in other cancers. In immunodeficient mice bearing human lymphoma cells, Bertolini *et al*(43) demonstrated that frequent low dose administration of cyclophosphamide suppressed EPC numbers and viability with concurrent inhibition of tumour growth possibly resulting from the anti-vasculogenetic effect consequent upon reduced EPC mobilisation. A phase two study administering celocoxib and low dose cyclophosphamide in patients with non-Hodgkin's lymphoma demonstrated that EPC numbers dropped and remained low in responders(44). Additional studies in lymphoma administering endostatin, a naturally occurring anti-angiogenic agent derived from type XVIII collagen, compared to chemotherapy resulted in greater inhibition of EPCs, bone marrow neovascularisation and increased tumour suppression(45, 46). Several studies failed to demonstrate a significant rise in EPCs in human cancer, including lymphoma, breast cancer(47) and gastric cancer(48), despite elevation of VEGF levels. It is suggested that VEGF and other mediator levels in these cancers are not elevated sufficiently to stimulate EPC production and recruitment. Within cancer,

EPCs are incorporated in to the neo-endothelium and contribute to tumour vessel formation(49) and tumour growth(50). EPCs have been shown to play an important role in the growth of tumours at both early and late stages(51). Their exact role as either markers of altered vascular integrity or direct contributors to the neoplastic process is not clear(32), and it is also not known whether their increase is the result of systemic endothelial activation in response to the cancer, a result of endothelial shedding, a response to cytotoxic chemotherapy or as a result of tumour induced mediators and growth factors. Gemcitabine treatment has been shown to affect VEGF-A and circulating endothelial cells but not EPCs(8). It may be that EPCs play an important role in tumour resistance, neo angiogenesis and facilitate metastasis due to both instructive (release of pro angiogenic cytokines) and structural (vessel formation, incorporation and stabilisation) functions(8). Pancreatic cancer is often diagnosed at an advanced stage and as discussed is highly resistant to chemotherapy treatment. As a therapeutic tool EPCs could potentially be used as a marker of anti-angiogenic therapy, tumour burden and growth and angiogenesis. In addition, EPCs may be a suitable marker to predict response to therapy, metastasis or recurrence. This potential makes EPC research promising in respect of monitoring the success of treatment and therapeutic options, particularly in APC.

All three EPC phenotypes were significantly increased in the trial patients but not control patients. There was no significant change in EPC levels seen in patients treated with gemcitabine monotherapy. In addition, EPCs ($CD45^-$, $CD31^+$, $CD34^+$ and $CD45^-$, $CD34^+$, $CD31^+$, $CD133^+$ phenotypes) were significantly increased compared with controls on comparison regression analysis. There was no significant increase in trial $CD45^-$, $CD31^+$ and $CD133^+$ EPCs compared to controls. These results demonstrate that EPCs are significantly increased in APC patients treated with intravenous ω-3FAs. Interestingly an increase in EPCs had been previously demonstrated following ω-3FAs administration in healthy volunteers(52, 53).

Theoretically if ω-3FA administration reduces the numerous pro-angiogenic mediators there would be a concomitant reduction in EPCs over the ω-3FA treatment period. However, ω-3FA administration appears to increase EPCs over the period of the treatment although the detail of the underlying mechanisms presently remains undetermined. It is possible that the reduction of pro-inflammatory mediators that result during ω-3FA treatment allows the expansion and recruitment of EPCs by alternative PAC mediators. Although reduction of the hypoxic stroma and inflammatory cuff seen in PAC should theoretically limit EPC expansion, there are clearly alternative mechanisms behind the EPC expansion seen with ω-3FA treatment and this requires further investigation.

An increase in EPCs may also confer a survival benefit and this was seen with an increase in EPCs in the trial patients with a large change in the level of EPCs compared to control patients. In EPCs with a $CD45^-$, $CD31^+$, $CD133^+$ and $CD45^-$, $CD31^+$ and $CD34^+$ phenotype there was a significant PFS and OS seen in trial patients with a large change of EPC levels compared with control patients. Additional analysis has observed a significant reduction in Platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) with omega 3 treatment over time(26). PDGF responders demonstrated a tendency towards improved OS and FGF responders a significantly improved PFS. The interaction between EPCs and growth factors in APC needs further investigation to determine if there is a therapeutic potential.

Conclusion

Administration of ω-3FAs with gemcitabine chemotherapy in APC results in a significant increase in the number of EPCs and there appears to be a survival benefit in patients with an increase in EPC numbers. This may be the result of a reduction in pro-inflammatory mediators however additional studies are required to ascertain the underlying mechanisms and pathways and the related clinical benefits.

Abbreviations

AA - Arachadonic acid

ALA – Alpha linoleic acid

APC – Advanced pancreatic cancer

DHA - Docosahexaenoic acid

EDTA - Ethylenediaminetetraacetic acid

EPA - Eicosapentaenoic acid

EPC – Endothelial progenitor cells

FACS – Fluorescence-activated cell sorting

FGF – Fibroblast growth factor

FSC – Forward scatter

PAC – Pancreatic adenocarcinoma

PBMC – Peripheral blood mononuclear cell

MCAM – Melanoma cell adhesion molecule

MHRA - Medicines and Healthcare Products Regulatory Agency

NSCLC – Non-small cell lung cancer

OS – Overall survival

ω-3FAs – Omega 3 fatty acids

ω-6FAs – Omega 6 fatty acids

PECAM-1 - Platelet and endothelial cell adhesion molecule 1

PDGF – Platelet derived growth factor

PFS – Progression free survival

RECIST - Response evaluation criteria in solid tumours

SSC – Side scatter

TNM – Tumour node metastasis

VEGF – Vascular endothelial growth factor

VEGFR-2 – Vascular endothelial growth factor receptor 2

Declarations

Ethics approval and consent to participate

Written consent was obtained from all study participants as per trial protocol and the local Ethics Committee and the Medicines and Healthcare Products Regulatory Agency (MHRA) approved both studies. All patients were assessed against trial protocol inclusion/exclusion criteria. The trial was registered with clinicaltrials.gov (Number:NCT01019382).

Consent for publication

Not applicable.

Availability of data and material-

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JI- Patient recruitment, sample analysis, data analysis, manuscript writing.

AA- Trial design, patient recruitment, sample analysis, data analysis, manuscript writing.

WC- Patient recruitment, sample analysis, data analysis, manuscript writing.

FR- Patient recruitment, sample analysis, manuscript writing.

JC- Patient recruitment, sample analysis.

CP- Trial design, patient recruitment.

LH- sample analysis, data analysis, manuscript writing.

JF - sample analysis, data analysis, manuscript writing.

JT- sample analysis, data analysis.

MSM - Trial design, patient recruitment, manuscript writing.

WS - Trial design, patient recruitment, manuscript writing.

ARD - Trial design, patient recruitment, manuscript writing.

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B.Braun supplied the investigated product Lipidem

Financial disclosure and conflicts of interest statement

None Declared

Conflicts of interest and funding sources

The investigational product was provided by BBraun.

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Figures

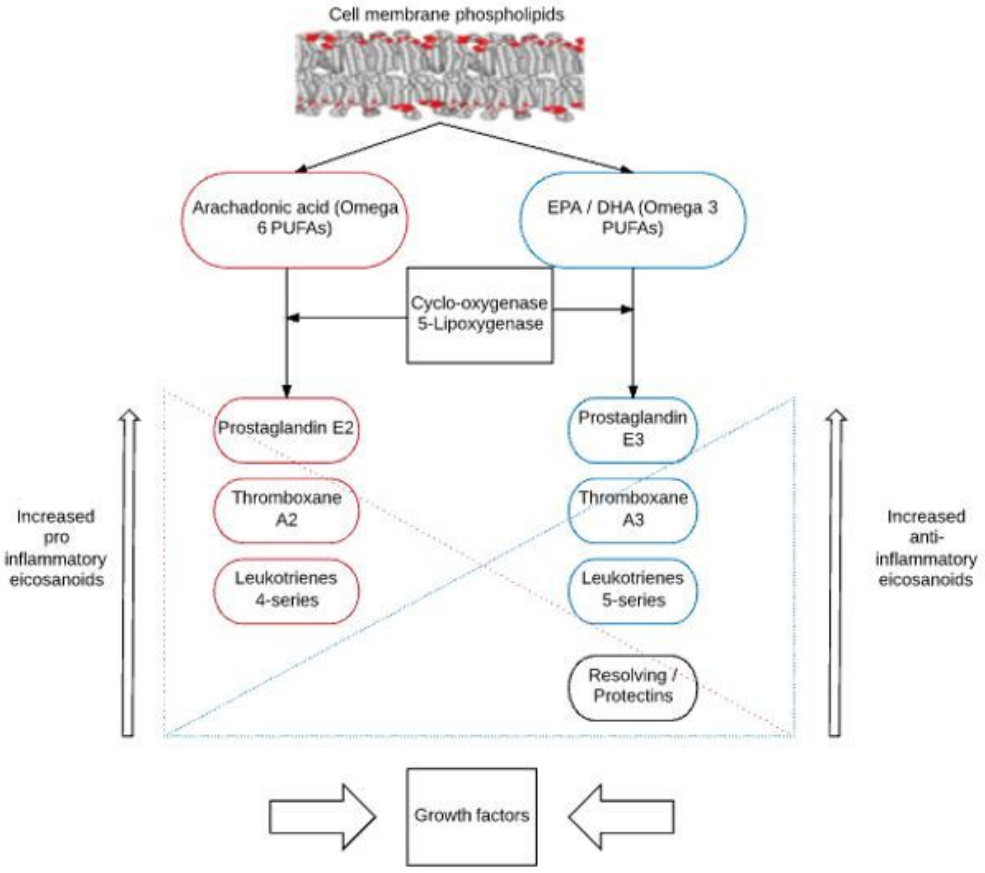

Figure 1

Eicosanoids derived from ω-3 FAs (EPA and DHA) and ω-6FAs (AA). They are metabolised by cyclooxygenase and lipoxygenase. The anti-inflammatory eicosanoids produced by ω-3FAs include the 3 series prostaglandins and thromboxanes and the 5 series leukotrienes. The pro inflammatory eicosanoids produced by ω-6FAs include the 2 series prostaglandins and thromboxanes and the 4 series leukotrienes. Increasing the amount of ω-3FAs improves the ω-3 FAs:ω-6FAs ratio and reduces the amount of ω-6FAs, particularly AA, present in the cell membrane and therefore the amount available for downstream pro inflammatory eicosanoid production. All these secondary mediators have either a general suppressive or enhancing effect on growth factors and cells in the inflammatory microenvironment.

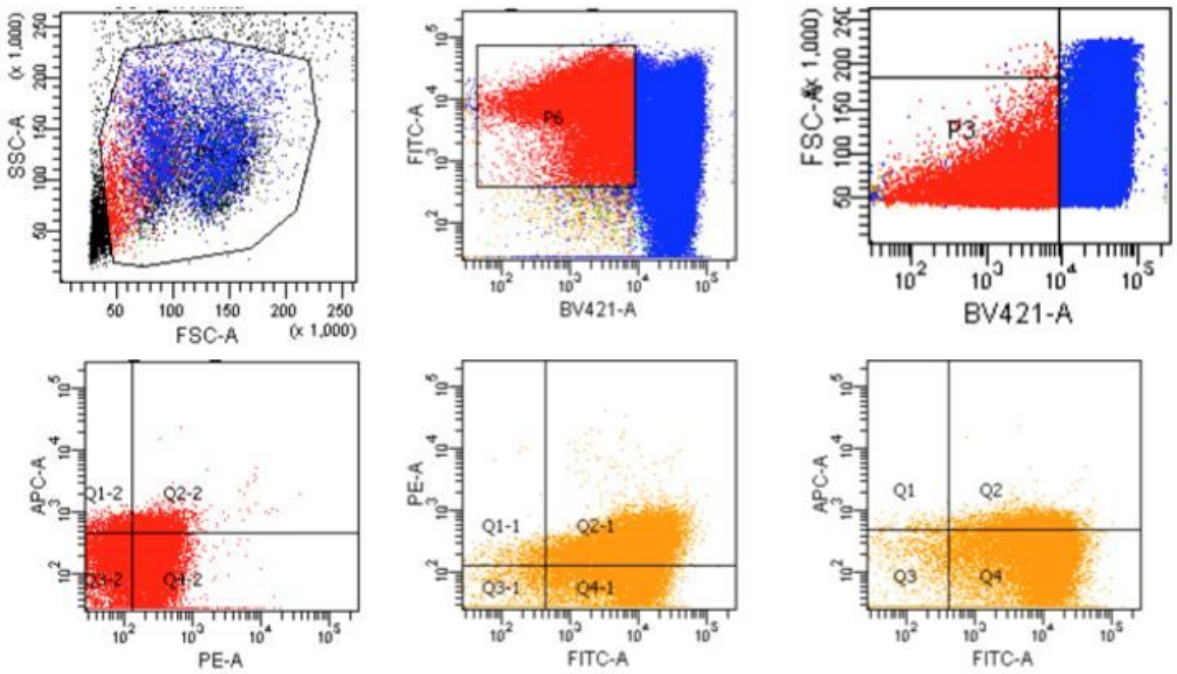

Figure 2

FACS schematic scatter plots of EPCs with a quadruple stain for CD45-, CD31+, CD133+ and CD34+ antibodies. The top left plot shows side scatter (SSC) versus forward scatter (FSC). A mean of 374,796 cells in the target population (P1) were analysed over the one hundred and thirty-four samples investigated. Three populations of EPCs were analysed. EPCs with a CD45-, CD31+, CD133+ and CD34+ phenotype are seen in Q2.2 (CD133+ (APC) and CD34+ (PE)), gated off P6 (CD31+ (FITC) & CD45- (R/B220 pacific blue). EPCs with a CD45-, CD31+and CD34+ phenotype are seen in Q2.1 (CD34+ (PE) and CD31+ (FITC)), gated off P3 (CD45- (R/B220 pacific blue). EPCs with a CD45-, CD31+and CD133+ phenotype are seen in Q2 (CD31+ (FITC) and CD133+ (APC)), gated P3 (CD45- (R/B220 pacific blue).

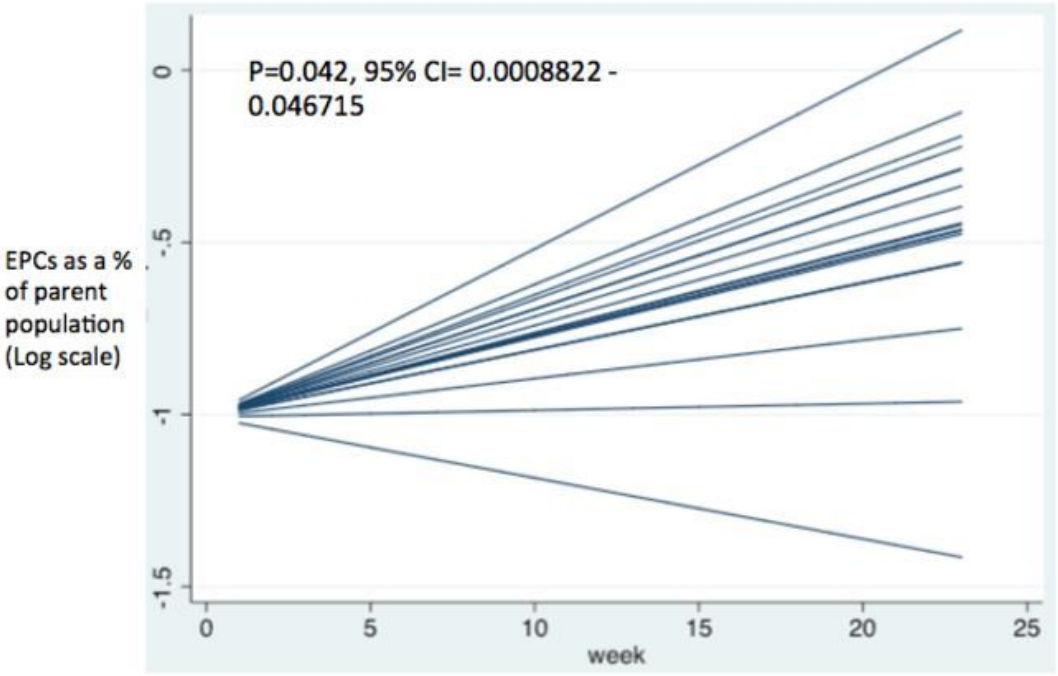

Figure 3

Output graph showing regression lines for individual patients plotted against time in weeks following mixed effects logistic regression analysis. EPCs levels are shown on the y axis and are a calculated as a percentage of the parent population (log scale), time in weeks is shown on the x axis. Each regression line represents a patient. One hundred and three time points in eighteen patients were analysed. There was a significant increase in EPCs over treatment (P=0.042, 95% CI= 0.0008 - 0.046).

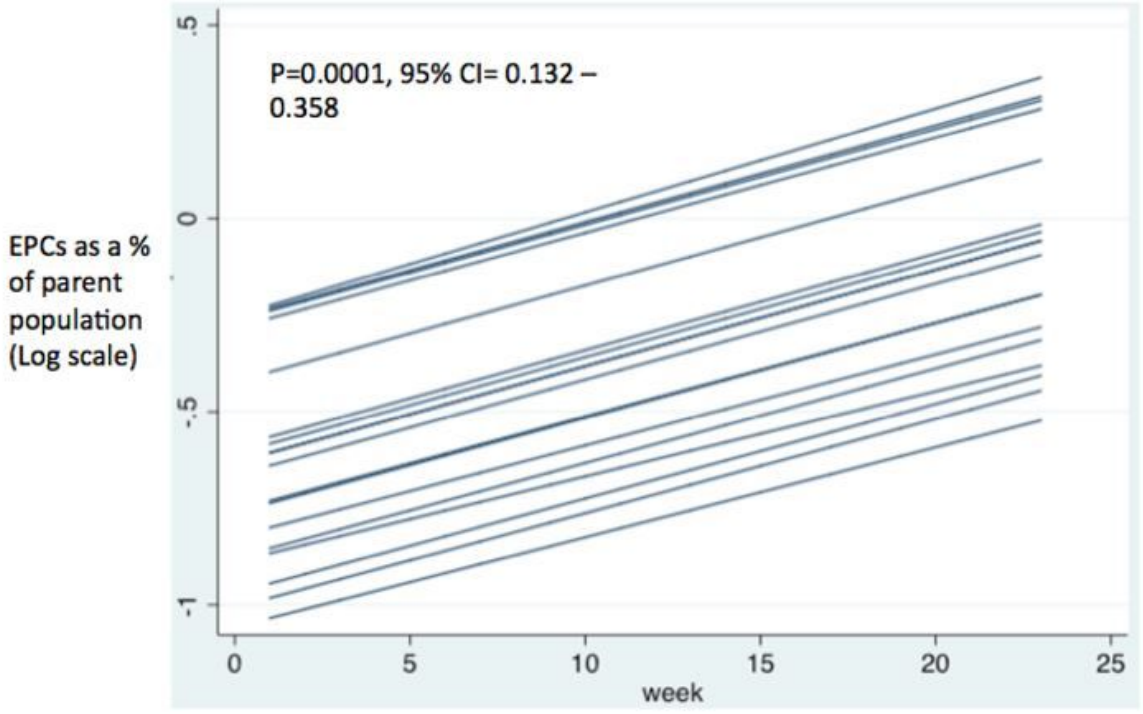

Figure 4

Output graph showing regression lines for individual patients plotted against time in weeks following mixed effects logistic regression analysis. EPCs levels are shown on the y axis and are a calculated as a percentage of the parent population (log scale), time in weeks is shown on the x axis. Each regression line represents a patient. One hundred and two time points in eighteen patients were analysed. There was a significant overall increasing trend in EPCs over time (P=0.0001, 95% CI= 0.132 - 0.385).

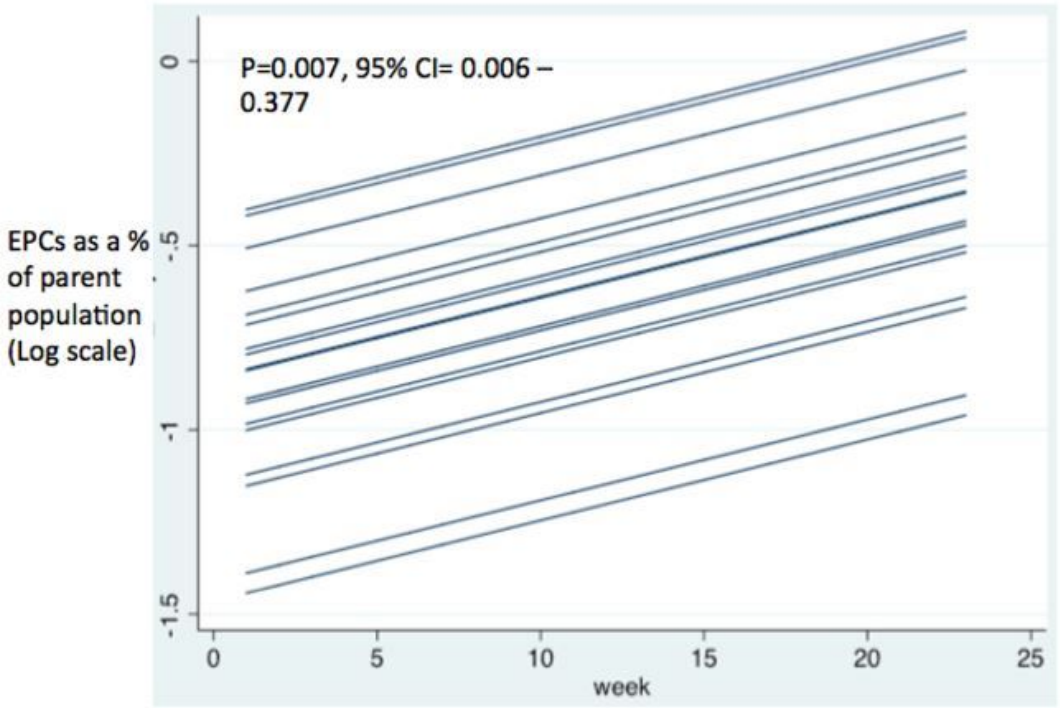

Figure 5

Output graph showing regression lines for individual patients plotted against time in weeks following mixed effects logistic regression analysis. EPCs levels are shown on the y axis and are a calculated as a percentage of the parent population (log scale), time in weeks is shown on the x axis. Each regression line represents a patient. Twenty-seven samples in nine patients were analysed. EPCs are calculated as a percentage of the parent population and plotted on a log scale. One hundred and three time points in eighteen patients were analysed. There is a significant increase in EPCs over time (P=0.007, 95% CI= 0.006 - 0.377).