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# Elucidating the Contribution of the Elemental Composition of Fetal Calf Serum Towards Antigenic Expression of Primary Human Umbilical Vein Endothelial Cells *In Vitro*.

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Running head: Serum Composition Mediates Cell Protein Expression

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## Abstract/Synopsis

One of the major obstacles in obtaining human cells of a defined and reproducible standard suitable for use as medical therapies is the necessity for fetal calf serum media augmentation in routine cell culture applications. Fetal calf serum (FCS) has become the supplement of choice for cell culture research as it contains an array of proteins, growth factors and essential ions necessary for cellular viability and proliferation *in vitro*. It is, however, a potential route for introduction of zoonotic pathogens and makes defining the cell culture milieu impossible in terms of reproducibility, as the precise composition of each batch of serum not only changes but is in fact extremely variable. This study determined the magnitude of donor variations in terms of elemental composition of FCS and the effect these variations had on the expression of a group of proteins associated with the antigenicity of primary Human Umbilical Vein Endothelial Cells (HUVECs), using a combination of inductively coupled plasma mass spectrometry (ICPMS) and flow cytometry. Statistically significant differences were demonstrated for a set of trace elements in FCS, with correlations made to variations in antigenic expression during culture. The findings question in detail, the suitability of FCS for the *in vitro* supplementation of cultures of primary human cells due to the lack of reproducibility and modulations in protein expression when cultured in conjunction with serum from xenogeneic donors.

## Keywords:

Elemental Analysis, Fetal Calf Serum (FCS), Flow Cytometry, *In Vitro*, Mass Spectrometry, Umbilical Vein Endothelial Cells

## Abbreviations:

FCS – Fetal Calf Serum  
 ICPMS – Inductively Coupled Plasma Mass Spectrometry  
 HUVEC – Human Umbilical Vein Endothelial Cells  
 PECAM – Platelet Endothelial Cell Adhesion Molecule  
 VCAM – Vascular Cell Adhesion Molecule  
 ICAM – Inter-Cellular Adhesion Molecule  
 vWF – Von Willebrand Factor

## Introduction

Evolution of human cell culture technologies has progressed through a diverse number of methods throughout the last century [1, 2]. Throughout this period, the goal of these studies was defined; to control the expansion and phenotype of primary human cells *ex vivo*. Refinement and improvement of cell culture techniques relied on the identification and reduction in the use of biological components required to fulfil these criteria [3, 4]. Whilst various studies across a multitude of cell types defined basal cocktails of salts, vitamins, sugars and other cell specific exogenous factors, all of these methods typically share one component, which without, advancement and understanding of *in vitro* cell culture would have proven to be an unobtainable objective: Serum [6]. Various species have been exploited as sources of serum for cell culture applications such as porcine [10], carpine [11] and human [12] with data demonstrating phenotype specific effects using one species of sera over another, such as myogenic and neurogenic cell cultures favouring equine serum [8, 9]. The sera of choice for most cell culture to date however is of bovine fetal calf origin (FCS), perhaps initially largely due to the ease of which it can be obtained, essentially as a by-product of the bovine meat production industry. For cell culture applications over one million bovine calves are harvested annually which equates to half a million litres of FCS destined for world wide tissue culture use [19].

FCS is an undefined m  le of proteins, cytokines and growth factors that provides non-specifically for cellular viability and proliferation *ex vivo*. It is harvested *in utero* from bovine fetuses at the point of sacrifice of pregnant female cattle and typically is used as a complement to basal culture media at concentrations of between five and twenty percent. FCS provides a straightforward candidate to facilitate the expansion of human cells by providing many molecules in excess that are required by cells to function correctly, be it identical or analogous to those found in human serum and interstitial fluid. FCS is a double edged sword however, adding simplicity and convenience to cell culture at a price. For cellular therapies and tissue engineering processes, FCS is completely unsuitable for the growth of cells destined for autologous or allogeneic implantation for several reasons: The possibility of contamination of human cell cultures with animal borne pathogens from bacteria, mycoplasma or prion origins and also the immunological adaptation of human cells to growth in xenogeneic serum [20].

The lack of constituent consistency leads to a lack of experimental reproducibility associated with FCS supplemented cultures, which can be attributed in many applications to variations in FCS composition across batches resulting from physiological and biochemical differences between donor animals [5]. This variation can occur as a result of gender [13], age [14], diet [15, 16], photoperiod [17, 18] and preparation protocol to name only a few of the possibilities [7]. Ultimately, this variation leads to inconsistencies in maintaining the phenotype and integrity of cells *in vitro*, plasticity particularly in multipotent cells such as mesenchymal stem cells is affected [49, 50]. Compositional variation across sera samples can be a combination of physiological differences in both protein and elemental concentrations, with the trace elements and their subsequent influence on cellular protein expression being the least investigated and understood of the two. The aim of this work was to investigate the significance of any elemental differences between sera samples and the resultant effects upon cellular behaviour and function.

The elemental component of FCS contains a surprisingly large representation from the periodic table of elements, with their precise role ranging from straightforward and vital to synergistic and complex. In this study we have compared the elemental composition across a number of FCS samples using inductively coupled plasma mass spectrometry (ICPMS). Furthermore, these sera were used in the culture of primary Human Umbilical Vein Endothelial Cells (HUVECs) to

determine the significance of elemental variation in FCS upon the antigenic expression of primary cells when cultured *ex vivo*. HUVECs were specifically chosen as a sentinel cell in this investigation due to their sensitivity to exogenous environmental stimuli, such as growth factors, underlying surface properties and purity of media/culture components. Although lineage committed, these cells can act as a reference against which other cells of greater plasticity can be compared, such as embryonic or bone marrow derived stem cells, or induced pluripotent cells. Additionally, when considered for *in vivo* clinical use these cells must remain in their optimal phenotypic state, providing a selective, non-thrombogenic barrier between the blood vessel lumen and the surrounding tissue, presenting in a direction aligned to blood flow to increase cell retention in an un activated state. Hence, when cultured *in vitro*, particularly when envisaging tissue engineered therapeutics, their phenotype must not be adversely altered through any *in vitro* culture phase.

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## Materials & Methods

### *Serum sampling*

FCS samples were obtained from a variety of commercial sources, denominated as donors 1-7. For all analysis these were used at a concentration of 20% (v/v) in conjunction with basal cell culture media.

### *Inductively coupled plasma mass spectrometry*

Samples were diluted 1:50 in 2% HNO<sub>3</sub> (v/v) (Romil SpA, GB) diluted to a working concentration using 18 MΩ ddH<sub>2</sub>O generated using a Purelab UHQ system (ELGA, UK). Standards were prepared by diluting Merck VI multi standard solution (MERK, UK) 1:1000 also using 2% HNO<sub>3</sub>. Further modifications were made to this standard solution such that it included Bromine and Sulphur PrimAg6 single element reference solutions (ROMIL, UK) and Phosphorus, Titanium and Rhodium Aristar single element standards (Merck, USA) all at a concentration of 1.0x10<sup>3</sup>ppm with the exception of Phosphorus which was included at 1.0x10<sup>4</sup>ppm due to the higher concentration of this element usually present in biological samples. Samples were analysed using the Totalquant platform (Perkin-Elmer, USA) on an Elan 6100 ICP-MS (Perkin-Elmer, USA). Duplicate dilutions were prepared from each sample (in 2% HNO<sub>3</sub>) and the Totalquant method reads each sample 5 times. 25ppb Rhodium was added to the sample 'online', just before the sample was nebulised, as an internal standard. Post analysis total sera elemental concentrations were normalised to reflect the concentration which the cells were subjected to when used at a concentration of 20% (v/v) during routine culture.

### *Cell Culture*

Primary HUVECs (Invitrogen, UK) were maintained on conventional plasma-treated tissue culture polystyrene (TCP) T25 flasks pre-coated with 0.1% gelatin. Prior to passage, cell culture media was removed from the tissue culture vessel and resultant adherent cells washed with PBS for 5 minutes to remove residual serum proteins. PBS was replaced by a solution of 5g porcine trypsin, 2g EDTA in 100mL of 0.9% sodium chloride (Sigma-Aldrich, UK) diluted to a working concentration of 10% (v/v) using PBS. Trypsinisation was performed at 37°C until approximately 75% of cells became detached from the substrate, observed by transmitted light microscopy. Cell/trypsin suspension was then diluted in an equal volume of basal cell culture media (1g/L glucose DMEM), pre-warmed to 37°C, containing 20% fetal calf serum 10,000 U/ml penicillin and 10 mg/ml streptomycin in 0.9% sodium chloride (Sigma Aldrich, UK). Diluted trypsin/cell suspension was then spun at 1.5x10<sup>3</sup> rpm for 5 minutes at 4°C to retrieve the cells, and the resultant cell pellet resuspended in an appropriate volume of cell culture media. This solution was then distributed throughout the desired number of tissue culture vessels and diluted further in a defined volume of cell culture media to permit long term culture.

Culture media was refreshed every 3<sup>rd</sup> day by aspiration of spent media followed by replacement using fresh cell culture media, pre-warmed to 37°C. This process was repeated until cells attained a density of approximately 2.0x10<sup>4</sup> cells/cm<sup>2</sup>, at which point passaging was repeated. All cells were reseeded at 6.0x10<sup>3</sup> cells/cm<sup>2</sup> and incubated at 37°C, in a humidified atmosphere, containing 5% CO<sub>2</sub>. HUVECs were maintained for three passages; using media containing one of the 7 experimental sera prior to antigenic analysis

### *Analysis of cells through flow cytometry*

HUVECs were detached from their substrate as outlined previously, suspended in 700μl PBS and divided between 7 flow cytometry tubes. 10μl of the appropriate antibody or isotype control was added, with one of the tubes containing cells only to assess the degree of cellular auto-fluorescence. FITC conjugated FACS antibodies for CD31, CD54, CD106 and von Willebrand

Factor (vWF) supplied by Becton Dickinson UK were used throughout. Samples were incubated for 30 minutes at 4°C before analysis using fluorescence-activated cell sorting (FACS) (FACSort, Becton Dickinson). The percentage positive specific antigen expression for each sample was obtained through comparison with an appropriate isotype control using the software platform WinMDI (<http://facs.scripps.edu/software.html>). For each antigen-serovar combination analysed, n=3.

*Statistical analysis*

Means and standard deviations were calculated for all quantitative data. Statistical analysis of variance (ANOVA) was used to test for significant differences in antigenicity between the serum samples. Post hoc, Tukey analysis ranked the data into homogeneous subsets. Pearson correlative analysis was performed between variation in elements and subsequent cellular antigen expression. Confidence levels were taken as 95%.

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## Results

### Elemental Composition of Sera

Sera samples were analysed using ICPMS with respect to a panel of 77 elements. A total of 40 elements were assessed to be significantly different between the samples. Figs. 1-3 illustrate the significant differences in a selection of these 40 elements when compared between the sera samples for; P-values for all of the significant elements are shown in Table 1.

### Cell Culture and Flow Cytometry

Significant positive expression of four antigens on HUVECs was determined through flow cytometry and compared between the sera donors. CD31, CD54 and CD106 demonstrated significant differences across the samples, producing statistically different subsets through post hoc ranking (P-values of 0.006, 0.004, and 0.022, respectively) (Fig.4.A-C). von Willebrand Factor (vWF), however, remained statistically similar between the sera groups (P=0.47) (Fig. 4.D).

### Correlative Statistical Analysis

Significant differences were established for a range of elements within the serum samples, and also for the cellular antigenic expression between the culture-sera conditions. Pearson correlative analysis was performed to establish the presence of relationships between these data. Analysis revealed significant correlations between antigenic expression and elemental composition for CD31, CD54 and CD106; however, there were no correlations found for vWF (Table 2).

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## **Discussion**

Significant differences in elemental components across the serovars were identified and extended consideration was allocated to elements with previous literature detailing their bioactive nature and known metabolic interactions.

### **Lithium**

Lithium physiology has been studied extensively for almost half a decade mainly due to its pharmacological interest in the treatment of physiological disorders. Lithium can substitute sodium or potassium on several transmembrane transport proteins, providing a shuttle for lithium influx into cells. The pathway for cellular efflux of lithium is more restricted, resulting in intracellular accumulation. The intracellular effects of lithium are several, including roles in the modulation of neuro-transmitter systems, neuropeptide systems and signal transduction pathways. For example, the cAMP signal transduction pathway, a second messenger system responsible for the conveyance of the intracellular consequences of a number of non-membrane permeable molecules such as adrenaline and glucagon, is a major target for intracellular lithium. Lithium inhibits adenylyl cyclase, a membrane bound protein responsible for cAMP synthesis, in a number of cell types. Although only present in minor concentrations when compared to more extensively studied trace cations such as calcium and magnesium, variations of statistical significance were observed in the concentration of lithium, an element with an extremely small therapeutic dose effect across the donor set (Fig.1.A.) [29].

### **Boron**

Boron, although less defined molecularly, has been shown to have a possible role in cellular processes as diverse as reproduction and embryogenesis. Studies using amphibian models demonstrated that frogs (*xenopus laevis*) maintained on a boron deficient diet exhibited atrophied testes, decreased sperm counts and sperm dysmorphology. Female frogs exhibited atrophied ovaries and impaired oocyte maturation. Additionally, More than 80% of embryos from frogs fed a boron-deficient diet for 120 days died before 96 hours of development. Similar data has also been reported using zebra fish (*danio rerio*). Recently, a role for boron in osteogenesis has been demonstrated. Rats maintained on a boron deficient diet showed a marked reduction in the healing of alveolar bone in experimental models. Vertebrae from rats subjected to boron deprivation (0.1 mg/kg diet) from conception to age 21 weeks were compared to vertebrae from rats fed supplemental boron (3 mg/kg diet). Boron deprivation decreased bone volume fraction and trabecular thickness. Fluctuations in this element across the sera samples were highlighted by a large increase in donor 7 (Fig.1.B.) [21].

### **Magnesium**

Magnesium is the main intracellular earth metal ion found unchaperoned in the cytosol. Magnesium is a cofactor in a vast multitude of enzymatic reactions and is especially important for enzymes that use nucleotides as cofactors or substrates. This is because, as a rule, it is the stereochemistry of the nucleotide complex with magnesium that supplies these enzymes with a geometrically compliant substrate. This is true for phosphotransferases and hydrolases such as several members of the ATPase family which are of central importance in the biochemistry of the cell, particularly in energy metabolism. In addition, magnesium is required for protein and nucleic acid synthesis, the cell cycle, cytoskeletal and mitochondrial integrity and for the binding of substances to the plasma membrane. Magnesium frequently modulates ion transport by pumps, carriers and channels and thereby may modulate signal transduction and the cytosolic concentrations of calcium and potassium. A large diversity in the concentration of this ion was observed across the fetal calf serum donor set (Fig.1.C.) [30].

### Phosphorus

Phosphorus is found abundantly as organic phosphate moiety in a plethora of bioactive intracellular signalling molecules including ATP and cAMP. Possibly the most fundamental function of phosphate is its rate limiting role in glycolysis facilitating the formation of glucose-6-phosphate, which provides an example of just one critical intracellular pathway which is mediated by phosphorylation. A second fundamental example which highlights the physiological importance of intracellular phosphate is in the phospho-diester bonding which occurs throughout polynucleic acids such as DNA and RNA, providing molecular strength and precise geometry. On a macroscopic level, phosphorus associates with calcium in the formation of hydroxyapatite, the molecule responsible for giving bone its mechanical strength. This element was also found to have significant differences in concentrations throughout the donor sample set (Fig.1.D.) [34].

### Sulphur

The main function of sulphur biochemically is its role in the thiol (-SH) linkage of the specialised peptide; glutathione. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the -SH group of the cysteine moiety of glutathione is able to donate a reducing equivalent ( $H^+ + e^-$ ) to unstable molecules. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). This reaction is critical for intracellular detoxification by removal of reactive oxygen species such as free radicals and peroxides. Furthermore, this reaction is capable of maintaining exogenous antioxidants such as ascorbate and tocopherol in their reduced states. Glutathione also plays a role in the immune system by enhancing the proliferation of lymphocytes and increasing the efficacy of cytotoxic T and NK cells. In the experimental sample set of particular note was a significantly higher concentration of sulphur recorded in sera donor 7 (Fig.1.E.) [39, 40].

### Potassium

Potassium is the most abundant cation in adult human tissue and plays a fundamental role in the maintenance of osmotic potential in the cell through its transmembrane traffic between cytosol and interstitial fluid. Additionally, another critical cellular role of potassium is in nerve transduction. When a neuron fires, the cell wall instantaneously becomes permeable to sodium ions, which move into the cell, followed by, to maintain the membrane potential, an efflux of potassium ions, which is merely one example of the specialised electrophysiological role of the membrane transflux of this ion. Additionally, potassium plays a cofactorial role in numerous enzyme mediated reactions and has functions in the maintenance of intracellular pH. Fig.1.F. demonstrates the profile of concentrations of potassium across the experimental sera, which illustrates a significant increase of this element in donor 7.

### Titanium

Titanium was also considered for analysis, although defined physiological roles for this element are difficult to elucidate. Titanium has long been favoured in biomaterial and tissue engineering disciplines due to its influences on cellular spreading and adhesion, its mechanical compliance for orthopaedic devices in particular, and critically its excellent biocompatibility *in vivo*, due to the extremely stable insoluble layer of titanium oxide which forms instantly on the surface of pure titanium in air and which forms the tissue/material interface. Although no defined metabolic role could be elucidated from previous literature for this ion it is considered bioactive and to have an influence on cells. Fig.1.G. demonstrates the concentration of titanium in the

experimental sera further characterising a significantly higher concentration in sera obtained from donor 7 [40, 41].

### Vanadium

Vanadium is an element rarely referred to as having significant physiological function, however vanadium biochemistry is currently gaining global research momentum. In the form of vanadate, this ion plays a role as a cofactor for two crucial enzymes; Ribonuclease; an enzyme that catalyses the cleavage of RNA, and ATPases; enzymes responsible for cleavage of phosphate-anhydride bonds which play a role in a number of cellular metabolic processes. The role of vanadate in enzymology revolves around its function in transition state stabilisation; maintenance of an energetically unfavourable state of an enzyme/substrate complex during catalysis. Vanadium has also been demonstrated to synergistically enhance the function of insulin in diabetic, hyperglycaemic animal models, although the mechanism for this action is undefined. Both donors 3 and 7 were found to have significantly elevated concentrations of vanadium in their sera (Fig.1.H) [42, 43].

### Chromium

Chromium is hypothesised to be an essential trace element. This metal affects carbohydrate and lipid metabolism via the action of insulin although the precise mechanism for this mediation is not fully understood. The oligopeptide chromodulin binds chromic ions in response to an insulin-mediated chromic ion flux, and chromium saturated chromodulin can associate with insulin-stimulated insulin receptors, activating the receptor's tyrosine kinase activity. Thus, chromodulin appears to play a role in an autoamplification mechanism in insulin signalling. Sera obtained from donor 7 demonstrated a significant increase in the concentration of chromium (Fig.2.A.) [24].

### Manganese

Manganese plays many roles in biological systems ranging from acting as a simple Lewis acid catalyst to potentially transversing several oxidation states to carry out cofactorial roles in enzyme mediated catalysis. Possibly the most documented role of manganese is as a cofactor of arginase, a metallohydrolase which converts arginine to urea and ornithine, a component of the uric acid cycle; the physiological series of reactions of which the result is the detoxification of ammonia by its conversion to urea. Variations in the concentration of manganese were recorded across the sample set with the most significant occurring in sera obtained from donor 4 (Fig.2.B.) [31].

### Iron

Iron is essential for DNA synthesis, respiration, ATP synthesis through iron dependent enzymes and control of apoptosis. The levels of iron in the cell must be delicately balanced, as iron over loading leads to free radical damage by means of superoxide driven Fenton chemistry. Iron is also the inorganic component of Heme (iron protoporphyrin IX) which exists as prosthetic group in several proteins, which include respiration cytochromes, gas sensors, P450 enzymes, catalases, peroxidases, nitric oxide synthases, guanyl cyclases, and transcription factors. Hemin (the oxidized form of Heme) is an essential regulator of gene expression and growth promoter of hematopoietic progenitor cells. Heme is transported into hematopoietic cells and enters the nucleus where it activates gene expression by removing transcriptional potential repressors, such as Bach1, from enhancer DNA sequences. Variations in the concentrations of iron in the experimental sample set are highlighted by a significant increase in sera from donor 7 (Fig.2.C.) [27, 28].

### Copper

Copper features extensively in the catalysis of oxidation-reduction reactions involving oxygen or various oxygen radicals. Possibly the most classical example of a copper dependent enzyme is cytochrome C oxidase, and enzyme with 2 copper prosthetic sites and the terminal enzyme of the electron transport chain in mitochondria. Ultimately, the site where oxygen is utilised in respiration, the reaction catalysed is the reduction of  $O_2$  to  $2H_2O$ . The change in mitochondrial membrane potential innervated as a result of this reaction acts as an initiator for the enzyme ATP synthase to generate ATP. A further example of a critical copper requiring enzyme is superoxide dismutase which usually contains copper and zinc moieties. Copper-zinc superoxide dismutase is distributed widely in the cytosol of eukaryotic cells, where it carries out the dismutation of two singlet oxygen atoms to molecular oxygen and hydrogen peroxide, an important cytosolic detoxification process for the removal of superoxide radicals. This study demonstrated a similar profile of copper concentrations to that observed with iron, showing a significant increase in sera from donor 7 (Fig.2.D.) [25].

### Zinc

Zinc is known to participate in a wide variety of physiological processes including protein, nucleic acid, carbohydrate and lipid metabolism. Zinc has a cofactorial role in a multitude of enzymes including alcohol dehydrogenases, aldolases, alkaline phosphatases, carboxypeptidases, leucine amino peptidases, DNA and RNA polymerases, isomerases and transphosphorylase. Zinc is crucial for proteins or enzymes that interact directly with DNA, often these molecules contain a 'zinc finger'. A zinc finger is a large super-family of protein domains that bind polynucleic acids. A zinc finger consists of two antiparallel  $\beta$  strands, and an  $\alpha$  helix which are coordinated by zinc ions linking charged histidine and cysteine residues. The zinc ion is crucial for maintaining the spatial stability of this domain type. In the absence of the metal ion the domain unfolds removing the precise spatial conformation required for DNA major groove interaction and sequence specific DNA recognition by transcription factors. The concentration of zinc across the experimental sera set was found to be varied with significantly higher concentrations occurring in donor 5 and 7 (Fig.2.E.) [44].

### Gallium

Gallium has been demonstrated to have a biological efficacy although the exact mechanisms that are employed to mediate its functions are unknown. Presently, gallium is known to have immunomodulatory roles in the inhibition of T-cell proliferation and also causes inhibition of MHC class II molecules on macrophage cells. Gallium has been shown to interact directly with the iron chaperone protein, transferrin. It has been demonstrated that the gallium transferrin complex has the potential to inhibit the proliferative response of peripheral blood mononuclear cells. Furthermore the gallium bound transferrin was shown to inhibit the IL2 mediated activation of T cells, in addition to increasing their surface expression of the transferrin receptor, demonstrating a convoluted yet significant role in mediation of the inflammatory response. The concentrations of gallium in sera were demonstrated to be small, although differences found to be significant were observed across the data set (Fig.2.F.) [26].

### Selenium

Selenium is bioactive in the form of selenoproteins, a protein superfamily in which the sulphur moiety of a cysteine residue has been substituted by a selenium ion. The most abundant selenoprotein family are the glutathione peroxidases which are involved in the intracellular reduction of lipid peroxides and intracellular hydrogen peroxide. Selenium has also been demonstrated to have a role in the initiation of apoptosis in cultured cells, possibly through the initiation of single strand DNA breaks, although the exact mechanisms are still undefined.



Additionally, selenium has been demonstrated to have a role in the inhibition of cell proliferative status through modulation of NF- $\kappa$ B signalling pathways. It also forms part of the active site of thioredoxin reductase; an enzyme involved in the reduction of insulin. The sera samples demonstrated varied and statistically significant spread in their concentrations of selenium (Fig.2.G.) [36, 37].

#### Bromine

Bromine has a number of undefined physiological roles. Possibly the most understood is in the coordination of sleep through 2-Octyl  $\gamma$ -bromoacetoacetate, an endogenous compound originally isolated from human cerebrospinal fluid. This molecule inhibits fatty acid amide hydrolase; an enzyme that degrades neuromodulatory fatty acid amides, particularly oleamide, thus initiating sleep. The concentration of bromine across the experimental sera set was diverse with significantly higher values observed from donors 3 and 5 (Fig.2.H.) [22, 23].

#### Rubidium

Rubidium has an undefined role although has been demonstrated to enter cells using the same protein pump system used to transport potassium ions. The concentrations of this element were found to be significantly higher in sera from donor 7 (Fig.3.A.) [35].

#### Strontium

The physiological nature of the actions of strontium intracellularly are unknown, however, it is found distributed widely throughout a diverse range of tissues *in vivo*, particularly in bone. Significant increases in the concentration of strontium in sera were observed in donors 5 and 7 (Fig.3.B.) [38].

#### Molybdenum

Molybdenum represents an important cofactorial trace element involved in the structure of certain enzymes catalysing intracellular redox reactions. Most crucially; xanthine oxidase, the enzyme responsible for the catalysis of the oxidation of xanthine to uric acid contains molybdenum. A further example is xanthine dehydrogenase, a molybdenum-containing enzyme involved in purine catabolism. Although overall concentrations of molybdenum in serum was low, donor 4 reported significant reductions in the concentrations of this element (Fig.3.C.) [32, 33].

Antigenic expression of CD31, CD54 and CD106 was significantly altered in the HUVECs cultured in conjunction with the different serovars; only vWF expression remained statistically similar trans-donor. CD31 (PECAM-1; Platelet Endothelial Cell Adhesion Molecule-1) plays a role in mediating cell-cell adhesion and is a key biomarker for the occurrence of angiogenesis and endothelialisation [45]. CD54 (ICAM-1; Intercellular Adhesion Molecule) also has importance in stabilising cell-cell adhesion and interactions, and is also involved in activation processes and cell signalling [46]. CD106 (VCAM-1; Vascular Cell Adhesion Molecule-1), promotes cell adhesion, interactions and is a constituent part in signal transduction [47]. vWF is of critical importance in protein binding, wound healing and blood coagulation [48]. Considering the roles of these molecules biochemically, CD31, CD54 and CD106 are responsible modulating the interaction and adhesion of the endothelial cells immediate environment; the surrounding cells, exogenous factors and substrate, thereby presenting a palette of proteins indicative of an endothelial antigenicity. Hence, these were altered through culturing the cells in the varied serum-containing media, significantly altering the cellular protein expression.

Statistically, it was possible to demonstrate both differences in elemental/ionic composition of fetal calf serum obtained from a group of unrelated donors (Figs.1-3. & Table 1.), and furthermore cellular protein expression of HUVEC cells cultured in conjunction (Fig.4.). Correlative analysis revealed relationships between a subset of elements and the reported modulations in antigenic responses, particularly those associated with cellular activation and adhesion (Table 2.). This emphasises the biochemical synergy between elementary components of the fetal calf serum moiety and cells cultured therein. Considering the cofactorial role of several of the discussed elements in enzyme catalysed reactions it would be fair to suggest a rate limiting role of elemental composition of fetal calf serum on the action of a plethora of proteins within this undefined animal product. This, therefore, forms a basis for future research towards appropriate serum substitutes, determining the crucial inclusion of elemental components within any study; these trace elements, involved in complex multiple biochemical pathways, showed potential to produce fundamental associated responses in the cells, previously attributed in the majority to the proteinaceous component.

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## Conclusions

Statistically significant concentration differences were identified in a number of key regulatory ions in the elemental composition of sera obtained from different fetal bovine donors as determined using ICPMS. Tukey post hoc analysis provided a basis for the ranking of the sera donors into groups with statistically similar elemental compositions. FACs analysis of the antigenic changes in primary HUVECs cultured with different sera demonstrated differences in endothelial cell molecules associated with adhesion and activation. The biological significance of trans-donor variations in several key regulatory ions has been demonstrated using the endothelial cell protein expression as an *ex vivo* model. The question of the extent of the relationship between *in vitro* markers of cellular phenotype and *in vivo* functionality and the requirement for precise media elemental definition has been herein proven to be an important one. Therefore, further highlighting the necessity for the derivation of suitable serum free culture parameters for primary human cells, not solely for the generation of cells for clinical applications, but also for consistent cell maintenance from an experimental perspective.

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**Author Contributions**

Nicholas Bryan and Kirstie Andrews carried out the cell culture and flow cytometric analysis of cell antigenicity in addition to statistical analyses performed across all data sets. Michael Loughran performed the ICPMS analysis of the FCS serovars. Nicholas Rhodes and John Hunt were responsible for obtaining the funding from which this investigation was performed.

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## Figure and Table Legends.

*Fig.1. Comparison of the concentration of A; Lithium, B; Boron, C; Magnesium, D; Phosphorous, E; Sulphur, F; Potassium, G; Titanium, H; Vanadium in fetal calf serum at a concentration of 20% (v/v) in culture media from a range of unrelated donors. Error bars represent 1 standard deviation from the mean, n=5, Homogeneous subsets are represented by symbols, y=Donor, x=Concentration (ppb)*

*Fig.2. Comparison of the concentration of A; Chromium, B; Manganese, C; Iron, D; Copper, E; Zinc, F; Gallium, G; Selenium, H; Bromine in fetal calf serum at a concentration of 20% (v/v) in culture media from a range of unrelated donors. Error bars represent 1 standard deviation from the mean, n=5, Homogeneous subsets are represented by symbols, y=Donor, x=Concentration (ppb)*

*Fig. 3. Comparison of the concentration of A; Rubidium, B; Strontium, C; Molybdenum in fetal calf serum at a concentration of 20% (v/v) in culture media from a range of unrelated donors. Error bars represent 1 standard deviation from the mean, n=5, Homogeneous subsets are represented by symbols, y=Donor, x=Concentration (ppb)*

*Fig.4. Flow cytometric analysis of human umbilical vein endothelial cells cultured in conjunction with serum from a range of donors and their subsequent expression of: A. CD31; B. CD54; C. CD106; D. vWF. Statistical groupings of significant differences are indicated by differing symbols. Error bars represent 1 standard deviation from the mean, n=3.*

*Table.1. Trace elements found to contain statistical variations in their concentrations across the donor sera showing P values.*

*Table.2. Correlative statistical analysis of elemental composition and altered protein expression of HUVEC cells.*

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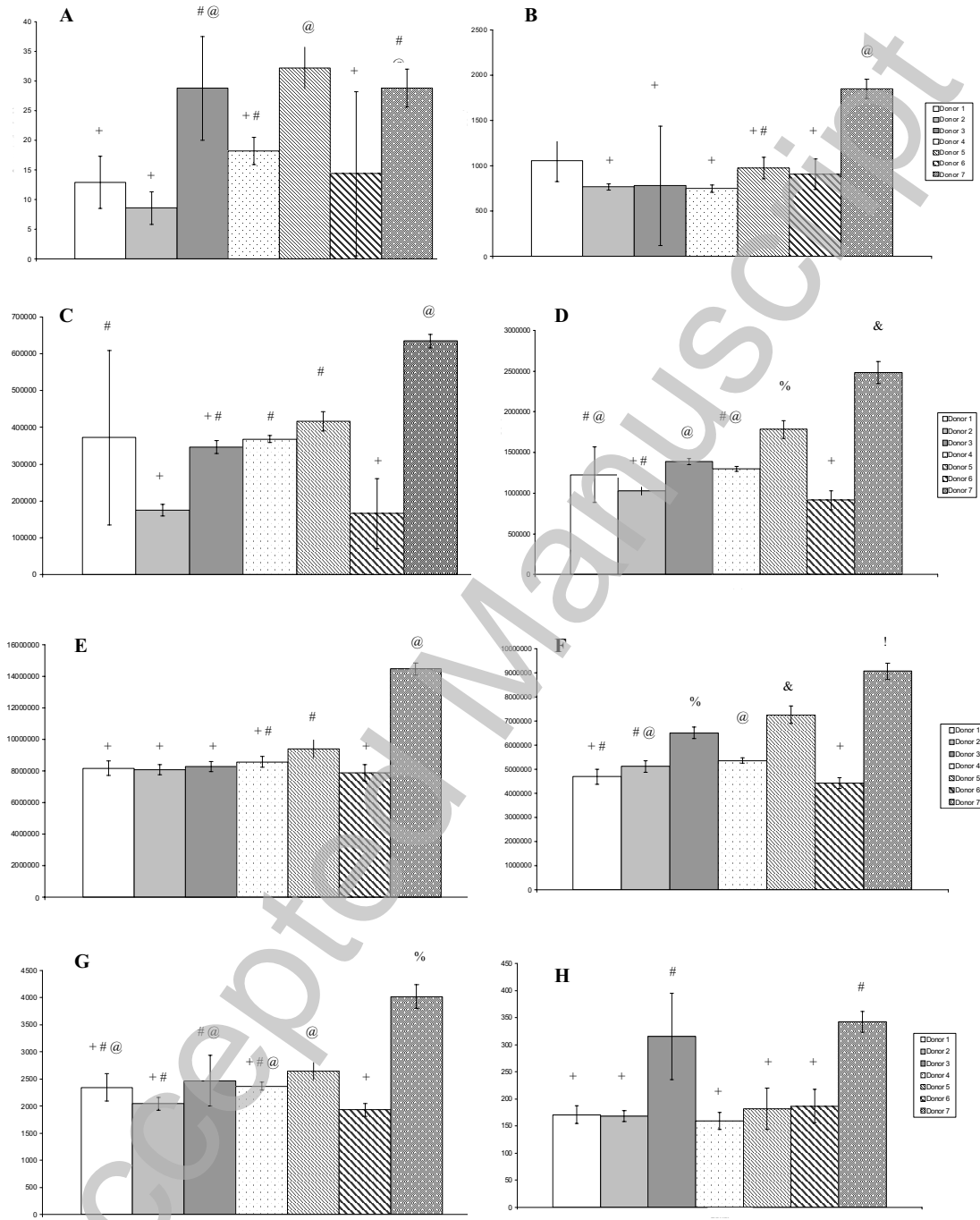


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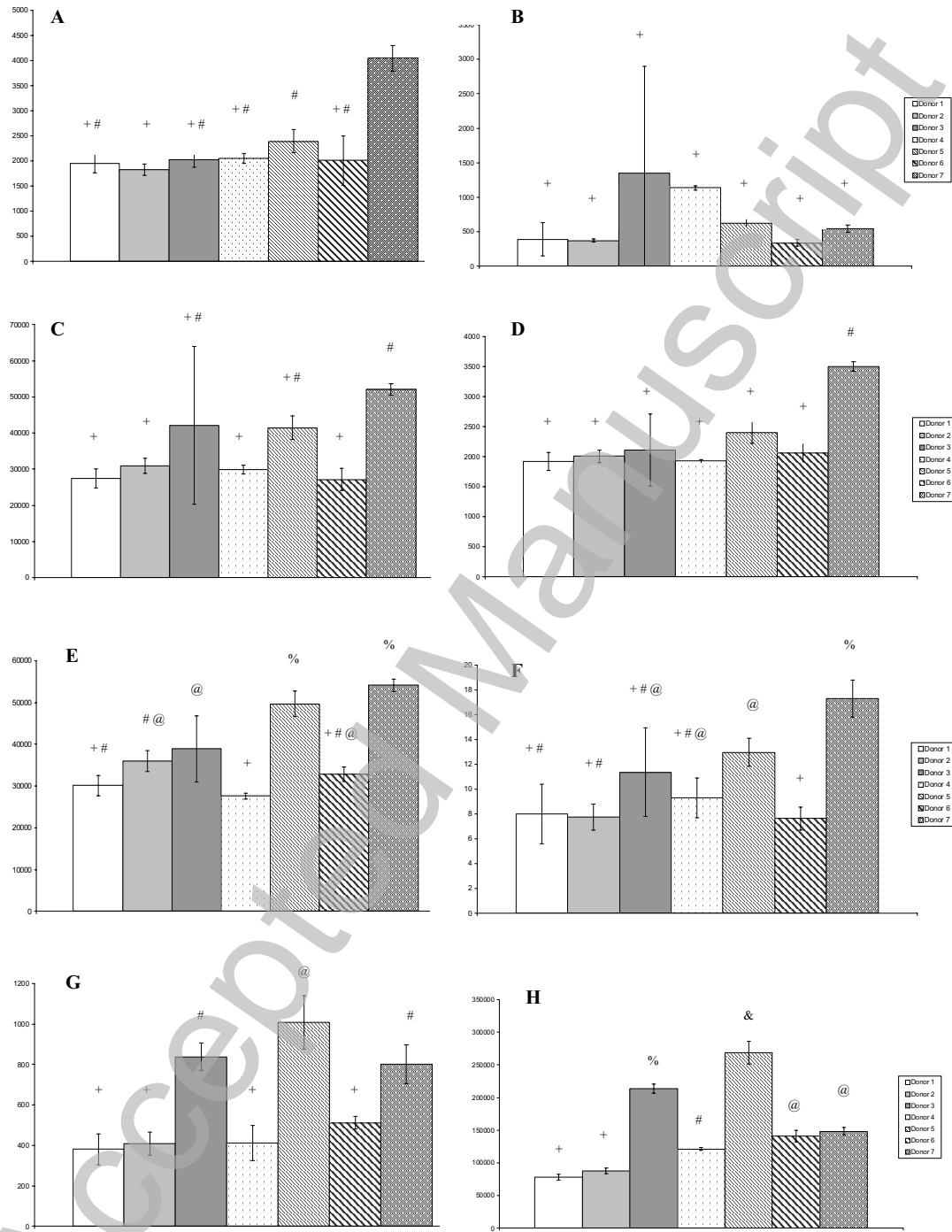


Fig. 2. Comparison of the concentration of **A**; Chromium, **B**; Manganese, **C**; Iron, **D**; Copper, **E**; Zinc, **F**; Gallium, **G**; Selenium, **H**; Bromine in fetal calf serum at a concentration of 20% (v/v) in culture media from a range of unrelated donors. Error bars represent 1 standard deviation from the mean, n=5, Homogeneous subsets are represented by symbols, y=Donor, x=Concentration (ppb)

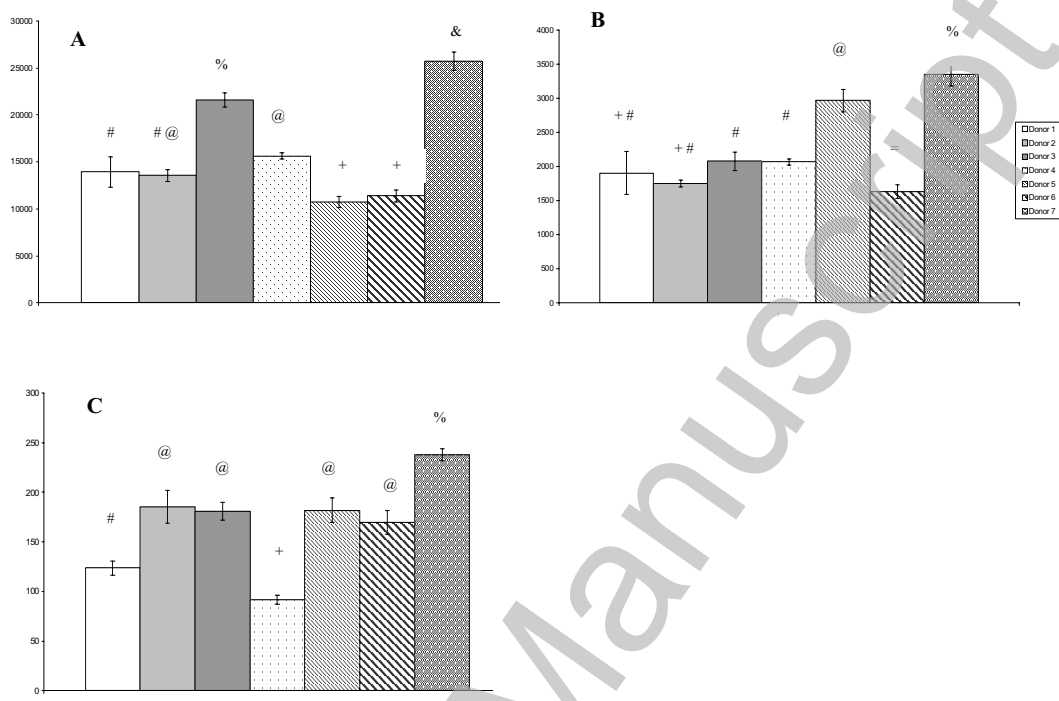


Fig. 3. Comparison of the concentration of *A*; Rubidium, *B*; Strontium, *C*; Molybdenum in fetal calf serum at a concentration of 20% (v/v) in culture media from a range of unrelated donors. Error bars represent 1 standard deviation from the mean, n=5, Homogeneous subsets are represented by symbols, y=Donor, x=Concentration (ppb)

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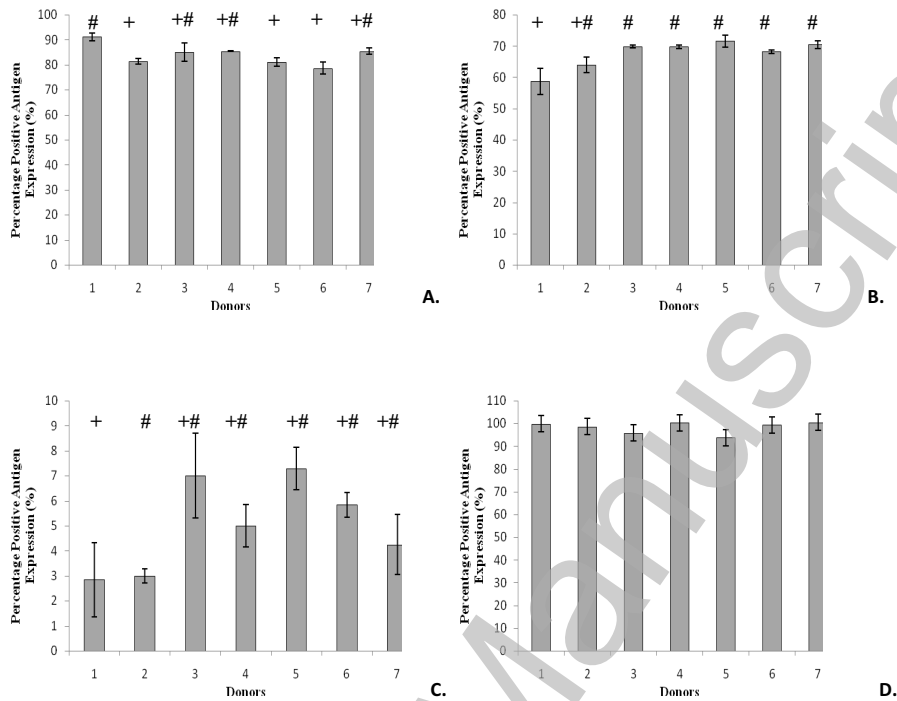


Fig.4. Flow cytometric analysis of human umbilical vein endothelial cells cultured in conjunction with serum from a range of donors and their subsequent expression of: A. CD31; B. CD54; C. CD106; D. vWF. Statistical groupings of significant differences are indicated by the differing symbols. Error bars represent 1 standard deviation from the mean, n=3.

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Table 1. ANOVA statistical analysis of differences in elemental composition between sera samples.

<b>Element</b>	<b>P</b>	<b>Element</b>	<b>P</b>	<b>Element</b>	<b>P</b>	<b>Element</b>	<b>P</b>
Lithium	0.000	Iron	0.000	Nitrogen	0.000	Palladium	0.045
Boron	0.000	Copper	0.000	Fluorine	0.000	Silver	0.042
Magnesium	0.000	Zinc	0.000	Neon	0.000	Antimony	0.000
Phosphorus	0.000	Gallium	0.000	Silicone	0.000	Tellurium	0.045
Sulphur	0.000	Selenium	0.000	Chlorine	0.000	Iodine	0.000
Potassium	0.000	Bromine	0.000	Scandium	0.000	Caesium	0.000
Titanium	0.000	Rubidium	0.000	Germanium	0.039	Lanthanum	0.031
Vanadium	0.000	Strontium	0.000	Astatine	0.000	Europium	0.005
Chromium	0.000	Molybdenum	0.000	Zirconium	0.003	Tungsten	0.000
Manganese	0.046	Carbon	0.000	Niobium	0.036	Thallium	0.000

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Table 2. Pearson correlative statistical analysis for relationships between variations in sera elemental composition and resultant cellular antigen expression.

<b>Antigen</b>	<b>Element</b>	<b>P</b>	<b>Pearson correlation</b>
CD31	Bromine	0.036	-0.496
	Tungsten	0.015	-0.564
CD54	Scandium	0.037	0.495
	Iron	0.032	0.507
	Zinc	0.043	0.481
	Selenium	0.005	0.627
	Bromine	0.004	0.638
	Tungsten	0.022	0.535
CD106	Bromine	0.046	0.476

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