Elucidating the contribution of the elemental composition of fetal calf serum to antigenic expression of primary human umbilical-vein endothelial cells in vitro

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Synopsis

One of the major obstacles to obtaining human cells of a defined and reproducible standard suitable for use as medical therapies is the necessity for FCS (fetal calf serum) media augmentation in routine cell culture applications. 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 the 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. The present 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, using a combination of ICPMS (inductively coupled plasma MS) 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 sera from xenogeneic donors.

Key words: elemental analysis, fetal calf serum (FCS), flow cytometry, human umbilical-vein endothelial cell (HUVEC), in vitro, mass spectrometry.

INTRODUCTION

The 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 the biological components required for fulfilling these criteria [3,4]. Although 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 without which advancement and understanding of in vitro cell culture would have proven to be an unobtainable objective: serum [5]. Various species have been exploited as sources of serum for cell culture applications such as pigs [6], goats [7] and humans [8] with data demonstrating phenotype-specific effects using one species of sera over another, such as myogenic and neurogenic cell cultures favouring equine serum [9,10]. The sera of choice for most cell cultures to date, however, are of bovine fetal calf origin [FCS (fetal calf serum)], perhaps initially largely due to the ease with which it can be obtained, essentially as a by-product of the bovine meat production industry. For cell culture applications, over 1 million bovine calves are harvested annually, which equates to half a million litres of FCS destined for worldwide tissue culture use [11].

FCS is an undefined mélée 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 killing of pregnant female cattle and typically is used as a complement to basal culture media at concentrations of between 5 and 20%. 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 xenogenic serum [12].

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 [13]. This variation can occur as a result of gender [14], age [15], diet [16,17], photoperiod [18,19] and preparation protocol to name only a few of the possibilities [20]. 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 [21,22]. 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 the present study was to investigate the significance of any elemental differences between sera samples and the resultant effects upon cellular behaviour and function.

The elemental composition 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 the present study, we have compared the elemental composition across a number of FCS samples using ICPMS (inductively coupled plasma MS). Furthermore, these sera were used in the culture of primary HUVEC (human umbilical-vein endothelial cells) to determine the significance of elemental variation in FCS upon the antigenic expression of primary cells when cultured \textit{ex vivo}. HUVEC were specifically chosen as a sentinel cell in this investigation owing 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 \textit{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 unactivated state. Hence, when cultured \textit{in vitro}, particularly when envisaging tissue engineered therapeutics, their phenotype must not be adversely altered through any \textit{in vitro} culture phase.

**MATERIALS AND METHODS**

**Serum sampling**

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

**ICPMS**

Samples were diluted 1:50 in 2% (v/v) HNO$_3$ (Romil SpA) diluted to a working concentration using 18 MΩ double-distilled water [generated using a Purelab UHQ system (Elga)]. Standards were prepared by diluting Merck VI multi-standard solution (Merck) 1:1000 using 2% HNO$_3$. Further modifications were made to this standard solution such that it included bromine and sulfur PrimAg6 single-element reference solutions (Romil) and phosphorus, titanium and rhodium Aristar single-element standards (Merck) all at a concentration of $1.0 \times 10^3$ p.p.m. with the exception of phosphorus that was included at $1.0 \times 10^2$ p.p.m. because a higher concentration of this element is usually present in biological samples. Samples were analysed using the Totalquant platform (PerkinElmer) on an Elan 6100 ICP mass spectrometer (PerkinElmer). Duplicate dilutions were prepared from each sample (in 2% HNO$_3$) and the Totalquant method reads each sample five times. We added 25 p.p.b. rhodium to the sample ‘online’, just before the sample was nebulized, as an internal standard. Post-analysis total sera elemental concentrations were normalized to reflect the concentration that the cells were subjected to when used at a concentration of 20% (v/v) during routine culture.

**Cell culture**

Primary HUVEC (Invitrogen) were maintained on conventional plasma-treated TCP (tissue culture polystyrene) T25 flasks precoated with 0.1% gelatin. Before passage, cell culture medium was removed from the tissue culture vessel and resultant adherent cells were washed with PBS for 5 min to remove residual serum proteins. PBS was replaced by a solution of 5 g of porcine trypsin and 2 g of EDTA in 100 ml of 0.9% sodium chloride (Sigma–Aldrich) diluted to a working concentration of $10\%$ (v/v) using PBS. Trypsinization was performed at $37^\circ$C until approx. 75% of the cells became detached from the substrate, observed by transmitted light microscopy. The cell/trypsin suspension was then diluted in an equal volume of basal cell culture medium (1 g/l glucose Dulbecco’s modified Eagle’s medium), prewarmed to $37^\circ$C, containing 20% (v/v) FCS, 10 000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% sodium chloride (Sigma–Aldrich). The diluted trypsin/cell suspension was then spun at $1.5 \times 10^3$ rev./min for 5 min at 4°C to retrieve the cells,
and the resultant cell pellet was resuspended in an appropriate volume of cell culture medium. This solution was then distributed throughout the desired number of tissue culture vessels and diluted further in a defined volume of cell culture medium to permit long-term culture.

Culture medium was refreshed every third day by aspiration of spent media followed by replacement using fresh cell culture medium, prewarmed to 37°C. This process was repeated until cells attained a density of approx. 2.0 × 10⁴ cells/cm², at which point passaging was repeated. All cells were reseeded at 6.0 × 10³ cells/cm² and incubated at 37°C, in a humidified atmosphere, containing 5% CO₂. HUVEC were maintained for three passages, using media containing one of the seven experimental sera before antigenic analysis.

Analysis of cells through flow cytometry
HUVEC were detached from their substrate as outlined previously, suspended in 700 μl of PBS and divided among seven flow cytometry tubes. Then, 10 μl of the appropriate antibody or isotype control was added, with one of the tubes containing cells only for assessing the degree of cellular autofluorescence. FITC-conjugated FACS antibodies for CD31, CD54, CD106 and vWF (von Willebrand factor) supplied by Becton Dickinson were used throughout. Samples were incubated for 30 min at 4°C before analysis using FACS (FACSort, Becton Dickinson). The percentage of positive specific antigen expression for each sample was obtained through a comparison with an appropriate isotype control using the WinMDI software platform (http://facs.scripps.edu/software.html). For each antigen-serovar combination analysed, n = 3.

RESULTS

Elemental composition of sera
Serum samples were analysed using ICPMS with respect to a panel of 77 elements. A total of 40 elements were assessed to be significantly different across the samples. Figures 1–3 illustrate the significant differences in a selection of these 40 elements when compared between the serum samples; 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 HUVEC was determined through flow cytometry and was compared between the serum donors. CD31, CD54 and CD106 demonstrated significant differences across the samples, producing statistically different subsets through post hoc ranking (P = 0.006, 0.004 and 0.022 respectively; Figures 4A–4C). vWF, however, remained statistically similar across the serum groups (P = 0.47; Figure 4D).

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 and sera conditions. A Pearson correlative analysis was performed to establish the 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).

DISCUSSION

Significant differences in elemental components across the serovars were identified and an extended consideration was allotted to elements with the literature detailing their bioactive nature and known metabolic interactions.
Figure 1  Comparison of the concentrations of (A) lithium, (B) boron, (C) magnesium, (D) phosphorus, (E) sulfur, (F) potassium, (G) titanium and (H) vanadium in FCS at a concentration of 20 % (v/v) in culture media from a range of unrelated donors. Error bars represent 1 S.D. from the mean, n = 5. Homogeneous subsets are represented by symbols; x-axis = donor, y-axis = concentration (ppb). Symbols (+, #, @, &, ! and %) represent statistical groupings as concluded by a Tukey's post-hoc ranking.
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 h of development. Similar results have 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 with vertebrae from rats fed on supplemental boron (3 mg/kg diet). Boron deprivation decreased bone volume fraction and trabecular thickness. Fluctuations in this element across the serum samples were highlighted by a large increase in donor 7 (Figure 1B) [24].

Table 2 Correlative statistical analysis of the elemental composition and altered protein expression of HUVEC

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Element</th>
<th>P value</th>
<th>Pearson correlation</th>
</tr>
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<tbody>
<tr>
<td>CD31</td>
<td>Bromine</td>
<td>0.036</td>
<td>−0.496</td>
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<tr>
<td></td>
<td>Tungsten</td>
<td>0.015</td>
<td>−0.564</td>
</tr>
<tr>
<td>CD54</td>
<td>Scandium</td>
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<td>Iron</td>
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<td>Tungsten</td>
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<tr>
<td>CD106</td>
<td>Bromine</td>
<td>0.046</td>
<td>0.476</td>
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Lithium

Lithium physiology has been studied extensively for almost half a decade, mainly because it is of pharmacological interest in the treatment of mental 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 numerous, 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 adenylate cyclase, a membrane-bound protein responsible for cAMP synthesis, in a number of cell types. Although only present in minor concentrations when compared with more extensively studied trace cations such as calcium and magnesium, variations of statistical significances were observed in the concentration of lithium, an element with an extremely small therapeutic dose effect across the donor set (Figure 1A) [23].

Magnesium

Magnesium is the main intracellular earth metal ion found unchaperoned in the cytosol. Magnesium is a cofactor in a vast multitude of enzymic 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 FCS donor set (Figure 1C) [25].

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 that is mediated by phosphorylation. A second fundamental example that highlights the physiological importance of intracellular phosphate is in the phospho-diester bonding that 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 (Figure 1D) [26].

Sulfur

The main function of sulfur biochemically is its role in the thiol (-SH) linkage of the specialized 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 reduced to its oxidized state (GSSG). This reaction is critical for intracellular detoxification by the removal of reactive oxygen species such as free radicals.
Figure 2  Comparison of the concentrations of (A) chromium, (B) manganese, (C) iron, (d) copper, (E) zinc, (F) gallium, (G) selenium and (H) bromine in FCS at a concentration of 20% (v/v) in culture media from a range of unrelated donors.

Error bars represent 1 S.D. from the mean, n = 5. Homogeneous subsets are represented by symbols; x-axis = donor, y-axis = concentration (ppb). Symbols (+, #, @, &, ! and %) represent statistical groups as concluded by a Tukey’s post-hoc ranking.
Serum composition mediates cell protein expression

**Figure 3** Comparison of the concentrations of (A) rubidium, (B) strontium and (C) molybdenum in FCS at a concentration of 20% (v/v) in culture media from a range of unrelated donors. Error bars represent 1 S.D. from the mean, n = 5. Homogeneous subsets are represented by symbols; x-axis = donor, y-axis = concentration (ppb). Symbols (+, #, @, &, ! and %) represent statistical groupings as concluded by a Tukey’s post-hoc ranking.

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 (natural killer) cells. In the experimental sample set, of particular note was a significantly higher concentration of sulfur recorded in serum donor 7 (Figure 1E) [27,28].

**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 the 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 specialized 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. Figure 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 owing 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 that forms instantly on the surface of pure titanium in air and that forms the tissue/material interface. Although no defined metabolic role could be elucidated from the literature for this ion, it is considered bioactive and to have an influence on cells. Figure 1(G) demonstrates the concentration of titanium in the experimental sera further characterizing a significantly higher concentration in serum obtained from donor 7 [28,29].
Vanadium
Vanadium is an element rarely referred to as having a significant physiological function; however, vanadium biochemistry is currently gaining global research momentum. In the form of vanadate, this ion plays the role of a cofactor for two crucial enzymes: RNase, an enzyme that catalyses the cleavage of RNA, and ATPases, enzymes responsible for the cleavage of phosphate–anhydride bonds that play a role in a number of cellular metabolic processes. The role of vanadate in enzymology revolves around its function in transition-state stabilization, i.e. the 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 (Figure 1H) [30,31].

Chromium
Chromium is hypothesized 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. Serum obtained from donor 7 demonstrated a significant increase in the concentration of chromium (Figure 2A) [32].

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 that converts arginine into urea and ornithine, a component of the uric acid cycle (the physiological series of reactions that result in the detoxification of ammonia by its conversion into urea). The variations in the concentration of manganese were recorded across the sample set with the most significant one occurring in serum obtained from donor 4 (Figure 2B) [33].

Iron
Iron is essential for DNA synthesis, respiration, ATP synthesis through iron-dependent enzymes and the control of apoptosis.
The levels of iron in the cell must be delicately balanced, as iron overloading leads to free radical damage by means of superoxide-driven Fenton chemistry. Iron is also the inorganic component of haem (iron protoporphyrin IX), which exists as a prosthetic group in several proteins, which include respiration cytochromes, gas sensors, cytochrome P450 enzymes, catalases, peroxidases, nitric oxide synthases, guanlyte cyclases and transcription factors. Haemin (the oxidized form of haem) is an essential regulator of gene expression and a growth promoter of haematopoietic progenitor cells. Haem is transported into haematopoietic 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 concentration of iron in the experimental sample set are highlighted by a significant increase in serum from donor 7 (Figure 2C) [34,35].

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, an enzyme with two copper prosthetic sites and the terminal enzyme of the electron transport chain in mitochondria, ultimately, the site where oxygen is utilized in respiration: the reaction catalysed is the reduction of O2 to H2O2. The change in mitochondrial membrane potential 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 cystosolic 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 serum from donor 7 (Figure 2D) [36].

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 superfamily of protein domains that bind polymeric acids. A zinc finger consists of two antiparallel β strands and an α-helix, which are co-ordinated 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 vary with significantly higher concentrations occurring in donors 5 and 7 (Figure 2E) [37].

Gallium
Gallium has been demonstrated to have a biological efficacy although the exact mechanisms that are employed to mediate its functions are unknown. At present, 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 (interleukin 2)-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 (Figure 2F) [38].

Selenium
Selenium is bioactive in the form of selenoproteins, a protein superfamily in which the sulfur moiety of a cysteine residue has been substituted by a selenium ion. The most abundant selenoprotein family is 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-κB (nuclear factor κB) signalling pathways. It also forms part of the active site of thioredoxin reductase, an enzyme involved in the reduction of insulin. The serum samples demonstrated varied and statistically significant spread in their concentrations of selenium (Figure 2G) [39,40].

Bromine
Bromine has a number of undefined physiological roles. Possibly the most understood is in the co-ordination of sleep through 2-octyl γ-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 (Figure 2H) [41,42].
Rubidium
Rubidium has an undefined role although it 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 serum from donor 7 (Figure 3A) [43].

Strontium
The physiological nature of the actions of strontium intracellularly is 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 (Figure 3B) [44].

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 the overall concentration of molybdenum in the serum was low, donor 4 reported significant reductions in the concentrations of this element (Figure 3C) [45,46].

Antigenic analysis of HUVECs
Antigenic expression of CD31, CD54 and CD106 was significantly altered in the HUVEC cultured in conjunction with the different sera; 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 of the occurrence of angiogenesis and endothelialization [47]. CD54 [ICAM-1 (intercellular adhesion molecule-1)] also has importance in stabilizing cell–cell adhesion and interactions, and is also involved in activation processes and cell signalling [48]. CD106 [VCAM-1 (vascular cell adhesion molecule-1)] promotes cell adhesion and interactions and plays a constituent part in signal transduction [49]. vWF is of critical importance in protein binding, wound healing and blood coagulation [50]. Considering the roles of these molecules biochemically, CD31, CD54 and CD106 are responsible for 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 FCS obtained from a group of unrelated donors (Figures 1–3, and Table 1) and, furthermore, cellular protein expression of HUVEC cultured in conjunction (Figure 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 emphasizes the biochemical synergy between elementary components of the FCS 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 FCS in 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 of cases to the proteinaceous component.

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’s post hoc analysis provided a basis for the ranking of the serum donors into groups with statistically similar elemental compositions. FACS analysis of the antigenic changes in primary HUVEC 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 a precise medium elemental definition has been herein proven to be an important one, further highlighting the necessity for 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.

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

Funding
This work was supported by a European Commission STEPS (Systems Approach to Tissue Engineering Processes and Products) project [grant number FP6-500465].
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Received 22 June 2010/1 September 2010; accepted 14 September 2010
Published as Immediate Publication 14 September 2010, doi 10.1042/BSR20100064