IMPACT OF HUMAN PLATELET LYSATE ON THE EXPANSION AND CHONDROGENIC CAPACITY OF CULTURED HUMAN CHONDROCYTES FOR CARTILAGE CELL THERAPY

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Abstract

High hopes have been pinned on regenerative medicine strategies in order to prevent the progression of cartilage damage to osteoarthritis, particularly by autologous chondrocyte implantation (ACI). The loss of chondrocyte phenotype during *in vitro* monolayer expansion, a necessary step to obtain sufficient cell numbers, may be a key limitation in ACI. In this study, it was determined whether a shorter monolayer expansion approach could improve chondrogenic differentiation. The effects of two supplement types, foetal bovine serum (FBS) and Stemulate™ (a commercial source of human platelet lysate), on the expansion and re-differentiation potential of human chondrocytes, isolated from five individuals, were compared. Chondrocytes were expanded with 10 % FBS or 10 % Stemulate™. Pellets were cultured for 28 d in chondrogenic differentiation medium and assessed for the presence of cartilage matrix molecules and genes associated with chondrogenicity. Stemulate™ significantly enhanced the proliferation rate [average population doubling times: FBS, 25.07 ± 6.98 d (standard error of the mean, SEM) *vs.* Stemulate™, 13.10 ± 2.57 d (SEM)]. Sulphated glycosaminoglycans (sGAG), total collagen and qRT-PCR analyses of cartilage genes showed that FBS-expanded chondrocytes demonstrated significantly better chondrogenic capacity than Stemulate™-expanded chondrocytes. Histologically, FBS-expanded chondrocyte pellets appeared to be more stable, with a more intense staining for toluidine blue, indicating a greater chondrogenic capacity. Although Stemulate™ positively influenced chondrocyte proliferation, it had a negative effect on chondrogenic differentiation potential. This suggested that, in the treatment of cartilage defects, Stemulate™ might not be the ideal supplement for expanding chondrocytes (which maintained a chondrocyte phenotype) and, hence, for cell therapies (including ACI).

Keywords: Human platelet lysate, Stemulate™, foetal bovine serum, human chondrocytes, cartilage, autologous chondrocyte implantation.

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Introduction

Damaged articular cartilage has a limited potential for healing and untreated cartilage defects often progress to osteoarthritis (OA) (Davies-Tuck *et al.*, 2008). Regenerative medicine strategies have been formulated to meet the challenge of preventing progression to OA; one such strategy is autologous chondrocyte implantation (ACI) (Nawaz *et al.*, 2014). ACI has evolved over 20 years, but chondrocytes are still the preferred cell choice. In brief, autologous chondrocytes are harvested by arthroscopy from a lesser weight-bearing area of the joint, expanded in monolayer culture and re-implanted into the damaged area under a natural or synthetic membrane by an open joint surgical procedure (Brittberg *et al.*, 1994). Robert Jones & Agnes Hunt (RJAH) Hospital, Oswestry and others report a high percentage of good to excellent clinical results (Bhosale *et al.*, 2009; Henderson *et al.*, 2003). Although there is limited long-term follow-up data, especially from the modern methods of ACI, positive clinical results are beginning to emerge. Nawaz *et al.* (2014) report a 78 % at 5 years and 51 % at 10 years ACI graft survival

for 827 patients. A robust economic model, based on a systematic review, indicates that ACI is cost-effective as compared with alternative procedures, providing long-lasting improvements in symptoms and eventually reducing the need for knee joint replacements (Mistry *et al.*, 2017; Web Ref. 1). Overall, the consensus is that ACI is more effective if used early following a cartilage injury and if it is the first attempt at a surgical intervention. Further refinement of ACI will ultimately enhance clinical outcomes and this, in part, is one of the goals of the present study.

To obtain sufficient cell numbers from small cartilage biopsies, the chondrocytes need to undergo *in vitro* expansion. The loss of chondrocyte phenotype, known as de-differentiation (Benya and Shaffer, 1982; Schnabel *et al.*, 2002), during *in vitro* expansion on tissue culture plastic is a key limitation of ACI. During de-differentiation, chondrocytes lose their rounded morphology and decrease gene and protein expression of collagen II and aggrecan, both key components of the articular cartilage extracellular matrix (ECM). Consequently, de-differentiation decreases the capacity of re-implanted chondrocytes to regenerate functional cartilage (Schulze-Tanzil, 2009). At the RJAH Hospital, Oswestry, most of the freshly isolated chondrocytes usually undergo two passages because de-differentiation is known to occur in as few as three passages (Kang *et al.*, 2007). Nevertheless, further minimising the conditions that promote chondrocyte de-differentiation may improve ACI.

Current standard protocols for chondrocyte expansion involve growth medium supplemented with foetal bovine serum (FBS), human autologous serum (AS) or serum-free medium. While FBS is used traditionally for *in vitro* chondrocyte expansion, an increase in demand and limited stocks are constantly increasing its price. FBS is a poorly defined mixture of components that can contain endotoxins, prion and viral proteins (Jochems *et al.*, 2002; Selvaggi *et al.*, 1997; van der Valk *et al.*, 2010). Current good manufacturing practice (GMP) guidelines state that animal products should be replaced by human alternatives, wherever possible, for translating into cell therapies (Giancola *et al.*, 2012; Unger *et al.*, 2008; World Health Organisation, 1992; World Health Organization, 1991). Human AS would seem the ideal solution, but patient-to-patient variability can negatively impact upon key parameters, such as chondrocyte growth and quality (Harrison *et al.*, 2000). Further, a relatively large volume of blood is required to perform chondrocyte expansion for ACI and this can be uncomfortable for the patients. A preferred alternative is defined serum-free growth media, but these media are supplemented with expensive growth factors that need to be replenished regularly during chondrocyte expansion. Given the issues described, other options are needed.

The use of platelet lysate for *in vitro* cell proliferation began in the 1980s (Choi *et al.*, 1980). Doucet *et al.*

(2005) describe the preparation and application of human platelet lysate (HPL) for expanding mesenchymal stem cells. Further studies show both autologous and allogeneic HPL to be superior to FBS in enhancing cell proliferation (Burnouf *et al.*, 2016; Hildner *et al.*, 2015; Hofbauer *et al.*, 2014; Kim *et al.*, 2015; Trojahn Kølle *et al.*, 2013). HPL eliminates the risk of cross-species contamination, solves standing ethical considerations and meets the GMP guidelines. In addition, it contains high levels of growth factors and cytokines and a range of vitamins and minerals that are also present in FBS and human AS (Hemeda *et al.*, 2014; Shih and Burnouf, 2015). HPL is created from large donor pools, reducing the variability observed with other serum types. However, there are limitations to HPL use, including the potential risk of contamination with human viruses, some of which are screened for, including HIV. Many studies show that 5-10 % (v/v) HPL is superior to 10 % (v/v) FBS in supporting cell expansion in several different cell types (King and Buchwald, 1984; Mojica-Henshaw *et al.*, 2013; Muraglia *et al.*, 2014; Reinisch *et al.*, 2007; Trojahn Kølle *et al.*, 2013; Witzeneder *et al.*, 2013). A HPL concentration of 10 % (v/v) supports cell proliferation at levels comparable to FBS (Burnouf *et al.*, 2016; Hemeda *et al.*, 2014; Hildner *et al.*, 2015; Hofbauer *et al.*, 2014). Only a handful of studies use HPL as an *in vitro* tool for human chondrocyte expansion (Gaissmaier *et al.*, 2005; Hildner *et al.*, 2015; Moreira Teixeira *et al.*, 2012; Muraglia *et al.*, 2014; Spreafico *et al.*, 2009). Even fewer investigate the recovery of chondrocyte phenotype, known as re-differentiation, in 3-dimensional (3D) culture when the HPL is excluded from the re-differentiation media (Hildner *et al.*, 2015). With ACI therapy in mind, it is important to ensure that the expanded chondrocytes have a chance to recover their chondrogenic phenotype in 3D culture without further exposure to relatively high levels of platelet-derived growth factors, which increase cell proliferation.

Stemulate™ (Cook Regentec, Indianapolis, IN, USA), a commercially available source of HPL, is prepared according to GMP guidelines, using large donor pools from accredited blood centres. Given that Stemulate™ is used to expand a range of cell types, including adipose-derived mesenchymal stem cells (MSCs) and bone-marrow MSCs, it could be an alternative supplement to increase chondrocyte proliferation for cartilage cell therapy (Badowski *et al.*, 2017; Juhl *et al.*, 2016; Mangum *et al.*, 2017; Riis *et al.*, 2016; Søndergaard *et al.*, 2017). Stemulate™ could reduce time in monolayer culture and reduce exposure to tissue-culture plastic, which in turn could reduce the risk of de-differentiation. The aim of the current work was to determine whether Stemulate™ could be a suitable alternative serum supplement to FBS for human chondrocyte expansion by assessing growth characteristics and cell morphology. Further, by using chondrogenic medium for both sets of chondrogenic pellets during re-differentiation in

3D culture, the aim was to assess the recovery of chondrocytes and determine their ability to produce ECM.

Materials and Methods

Chondrocyte isolation and monolayer expansion
Fully informed consent was obtained from five individuals undergoing total knee replacement surgery at RJAH Hospital, Oswestry, with ethical approval (11/NW/0875). Full depth cartilage was removed from macroscopically normal areas of the femoral condyle from three male and two female individuals with a mean age of 67 years (range: 55-75 years). Chondrocytes were isolated following an established protocol (Harrison *et al.*, 2000). Briefly, 100-300 mg of cartilage tissue were placed into 10 mL Dulbecco's modified Eagle's medium (DMEM)/F12 (11330-057; Gibco) containing 0.03 mg/mL deoxyribonuclease I (D4263; Sigma-Aldrich) and 0.83 mg/mL type XI collagenase (C9407; Sigma-Aldrich). Digestion was performed for 16 h in a T25 tissue culture flask (Sarstedt) at 37 °C and 5 % CO_2. The digest was filtered through a 0.2 μm cell strainer and chondrocytes were pelleted at 750 ×*g* for 10 min. After that > 98 % cell viability was verified using trypan blue (T8154; Sigma-Aldrich), the chondrocytes were plated into T175 tissue-culture flasks at a density of 5×10^4 cells/cm^2 in DMEM/F12 supplemented with 0.5 % (w/v) penicillin-streptomycin-amphotericin (17-745E; Lonza), 0.5 % (w/v) ascorbate (A4544; Sigma-Aldrich) and either 10 % (v/v) FBS (10500-064; Gibco) or 10 % (v/v) Stemulate™ (PL-SP-100; Cook Regentec). Media were replenished three times weekly. Once 80 % confluency was reached, the chondrocytes were passaged and re-seeded at a density of 5×10^4 cells/cm^2. Chondrocytes were cultured in monolayer until the end of passage 2.

Growth kinetics during monolayer expansion
To assess growth kinetics during monolayer expansion, population doubling times (PDTs) were recorded at each passage and calculated using the following formula (McAteer and Davis, 2002):

$$PDT = \frac{1}{multiplication\ rate}$$

$$\text{Multiplication rate} = \frac{3.32 \times [\log(final\ cell\ number) - \log(initial\ cell\ number)]}{time\ difference}$$

Re-differentiation in 3D pellet culture in chondrogenic medium
At the end of passage 2, the chondrocytes were detached and re-suspended in chondrogenic re-differentiation medium optimised from the Johnstone method (Johnstone *et al.*, 1998), comprising DMEM/F12 supplemented with 1 % (v/v) FBS, 1 % (v/v) non-essential amino acids (11140-035; Gibco), 1 % (v/v) L-glutamine (G7513; Sigma-Aldrich), 0.5 % (v/v) penicillin-streptomycin-amphotericin (17-745E;

Lonza), 0.5 % (w/v) ascorbate (A4544; Sigma-Aldrich), 1 % (v/v) insulin-transferrin-selenium (51500-056; Gibco), 20 ng/mL transforming growth factor-β1 (100-21; Peprotech), 10 nM dexamethasone (D4902; Sigma-Aldrich) and 3.2 μg/mL L-proline (P0380; Sigma-Aldrich). The chondrocytes (2.5×10^5 cells) were transferred to 1.5 mL Eppendorf® tubes and centrifuged at 750 ×*g* for 7 min, to create spherical pellets. Pellets were cultured in 500 μL of chondrogenic medium. Medium was replenished twice weekly.

Pellet processing for analyses
Pellets were assessed at day 0, 7, 14, 21 and 28. For biochemical assays, pellets (*n* = 5) were digested in 1 mg/mL proteinase K (25530-015; Ambion) in 100 mM ammonium acetate (09691; Sigma-Aldrich) for 2 h at 60 °C. Proteinase K was inactivated for 5 min at 100 °C and samples were stored at − 20 °C. For histology, pellets (*n* = 4) were placed onto Whatman filter paper (Whatman, Kent, UK), snap frozen in liquid-nitrogen-cooled n-hexane and stored at − 80 °C. For gene expression analysis, pellets (*n* = 3) were prepared for RNA extraction by homogenisation in 350 μL RLT lysis buffer containing 1 % (v/v) β-mercaptoethanol (M6250; Sigma-Aldrich) using a 21 G needle and syringe (1053393; QIAGEN) and stored at − 80 °C.

Pellet cell number
Cell number was determined using the PicoGreen® fluorescent DNA quantification kit (17916; Invitrogen), with lambda DNA as standard (1 ng/mL-1 μg/mL). Duplicate samples and standards were transferred to a 96-well plate and fluorescence was read at an excitation of 480 nm and emission of 520 nm using a FLUOstar Omega microplate reader (BMG, Ortenberg, Germany). Cell number was calculated using the widely reported value of 7.7 pg of DNA per chondrocyte (Kim *et al.*, 1988).

Total sGAG content in the pellets
The total sGAG was determined using 1,9-dimethylmethylene blue (DMMB) (Farndale *et al.*, 1986). All reagents were procured from Sigma-Aldrich. Chondroitin sulphate A from bovine trachea (C9819) was used as standard (0-200 μg/mL). In brief, 4 mg DMMB (341088) were diluted in 250 mL distilled water containing 0.76 g glycine (G5516) and 0.595 g sodium chloride (S5886) to a final pH of 3.0. Duplicate samples and standards were transferred to a 96-well plate and mixed with 200 μL of DMMB dye. Absorbance was read at 530 nm using a FLUOstar Omega microplate reader (BMG).

Total collagen content in the pellets
The total collagen content was determined by a hydroxyproline assay (Brown *et al.*, 2001). All reagents were procured from Sigma-Aldrich. Hydroxyproline standards (0-0.1 μg/mL; H5,440-9) were prepared in distilled water. 100 μL of each sample were hydrolysed

Table 1. Genes used in qPCR analysis. Genes were selected to determine the chondrogenic phenotype of the cells and if the chondrocytes had de-differentiated to a hypertrophic state.

Gene	Official name and species	Amplicon length	Entrez ID
Reference genes			
HPRT1	Hypoxanthine phosphoribosyltransferase 1 (human)	130 (NM_000194)	3251
TBP	TATA box binding protein (human)	132 (NM_001172085) 132 (NM_003194)	6908
Chondrogenic markers			
ACAN	Aggrecan (human)	62 (NM_001135) 62 (NM_013227) 62 (XM_001131727) 62 (XM_001131734) 62 (XM_00670419)	176
COL2A1	Collagen type 2 alpha 1 (human)	95 (NM_001844) / 95 (XM_006719242)	1280
SOX9	SRY (sex determining region Y)-box 9 (human)	112 (NM_000346)	6662
De-differentiation markers			
COL1A1	Collagen type 1 alpha 1 (human)	118 (NM_000088) 118 (NM_003257058) 118 (XM_005257059) 118 (XM_006721703)	1277
Hypertrophic markers			
ALK1/ACVRL1	Activin A receptor type II-like 1 (human)	74 (NM_000020)	94
COL10A1	Collagen type 10 alpha 1 (human)	91 (NM_000493)	1300

in 100 µL of 10 M hydrochloric acid (19068) for 16 h at 108 °C. On cooling, the samples were neutralised with 800 µL of 1 M sodium hydroxide (S5881). Duplicate samples and standards were transferred to a 96-well plate. 100 µL of oxidation buffer [600 mL isopropyl alcohol, 330 mL distilled water, 390 mL citrate/acetate buffer (1 L consists of 34 g sodium hydroxide (S5881), 34 g citric acid monohydrate (C7129) and 120 g sodium acetate trihydrate (32318-M)), pH 6.0] were used as a negative control. Oxidation solution [100 µL; 300 mg chloramine T (402869) dissolved in 50 mL oxidation buffer] was added to all samples and standards. The plate was incubated at room temperature for 5 min. Ehrlich's reagent [100 µL; 6 g p-dimethylaminobenzaldehyde (156477) dissolved in a mixture of 16 mL of 50 % perchloric acid (244252) and 52 mL of isopropyl alcohol] was added to each well and, then, the plate was sealed and incubated in a 60 °C water bath for 45 min. Absorbance was measured at 570 nm using a FLUOstar Omega microplate reader (BMG) and total collagen was calculated using the widely reported multiplication factor of 7 (Sims *et al.*, 2000).

qRT-PCR to assess gene expression in the pellets
mRNA was extracted using the QIAGEN RNeasy mini kit as per manufacturer's instructions. mRNA was reverse-transcribed in a Progene thermocycler (Techne, Staffordshire, UK) using a high-capacity cDNA reverse transcription kit (4368814; Applied Biosystems) following the manufacturer's instructions. qRT-PCR was performed on three

pellet replicates (from each patient) to determine gene expression relative to two reference genes, according to published guidelines (Bustin *et al.*, 2009): hypoxanthine-guanine phosphoribosyltransferase-1 (HPRT1 human; RT00059066) (Foldager *et al.*, 2009; Rushton *et al.*, 2014) and TATA box-binding protein (TBP; QT00000721) (Foldager *et al.*, 2009; Pombo-Suarez *et al.*, 2008) were selected based on low co-efficiencies of variance across both supplement types (data not shown). Several genes (Table 1) were chosen to determine the chondrogenic phenotype after re-differentiation. All genes, including reference genes, were procured from QIAGEN. Gene expression was measured relative to the reference genes and normalised to the cell number. Relative gene expression profiles were determined by the comparative C_T method (Schmittgen and Livak, 2008).

Histology of the frozen pellets
The frozen pellets were mounted onto pre-cooled chucks with optimal cutting temperature (OCT) compound (00.090.112; Sakura Finetek, Zoeterwoude, the Netherlands). All stains were procured from VWR International. 7 µm-thick sections were collected onto poly-L-lysine pre-coated slides (MBC-0102-54A; Cell Path, Newtown, UK) and stained with haematoxylin (350604T) and eosin (34197) or toluidine blue (340774Y), according to Roberts and Menage (2004). Sections were imaged by light microscopy (Leitz, Wetzlar, Leica Microsystems GmbH, Stuttgart, Germany) using ×6.3, ×25 and ×40 objective lenses. Images were captured using

a DS-Fi1 camera and analysed with NIS-Elements BR imaging software (Nikon). Images were white-balanced using Photoshop Elements (Adobe). To semi-quantify the toluidine blue staining intensity, four central images of each pellet were randomly selected and the integrated density was determined using ImageJ Software (NIH) according to the published methodology (Jensen, 2013; Owida *et al.*, 2017; Prasad and Prabhu, 2012).

Statistical analyses

All independent outcomes (PDTs, cell number, sGAG, total collagen, qPCR and staining intensity), patient variability and differences between the two supplement types over the time course were analysed using a multilevel model. Supplement type and time points were considered fixed variables and the patient was considered a random variable (random intercept). All qPCR data were log-transformed before the analysis, to make the data fit a normal distribution.

Cell number, sGAG and total collagen data ($n \geq 4$ per time point per patient, 5 patients in total) were expressed as mean ± standard error of the mean (SEM). Gene expression data ($n = 3$ per time point per patient, 5 patients in total) were shown as a box plot, with the box representing the second and third quartiles, the horizontal line inside the box representing the median and the whiskers either side of the box representing the lower and upper quartiles. All statistical analyses were performed with SPSS statistical software (version 24; IBM) and, for all analyses, $p < 0.05$ was deemed to denote statistical significance.

<div align="center">Results</div>

Stemulate™ positively influenced proliferation of chondrocytes in monolayer culture

For all 5 patients, the chondrocytes expanded in Stemulate™ (mean PDT: 13.10 ± 2.57 d) proliferated quicker than the chondrocytes expanded in FBS (mean PDT: 25.07 ± 6.98 d, $p = 0.050$). Patient variability was considered not statistically significant ($p = 0.48$) in affecting the PDT. Both sets of chondrocytes underwent less than 2 absolute population doublings throughout passages 1, 2 and 3 (Fig. 1). No statistically significant difference was measured in population doublings between the two sera supplements ($p = 0.527$) or at any passage ($p = 0.50$). Patient variability was considered not statistically significant ($p = 0.445$) in affecting population doublings.

Chondrocytes expanded in both FBS and Stemulate™ were seeded at an equal cell density upon chondrocyte isolation, after which both sets of chondrocytes showed a significant increase in cell number over time ($p < 0.001$; Fig. 2). Chondrocytes expanded in Stemulate™ demonstrated a significantly larger increase in cell number as compared to chondrocytes expanded in FBS ($p = 0.014$). Patient

variability was considered not statistically significant ($p = 0.296$) in affecting cell number in monolayer.

Fig. 3 shows the morphology of the human chondrocytes (day 4 of each culture) in the presence of either Stemulate™ or FBS during passage 0, 1 and 2. For Stemulate™-expanded chondrocytes, markedly more chondrocytes were present and they appeared to cluster together at all passages. For both supplement types, chondrocytes appeared elongated and flattened, with little difference in their cell shape at all passages.

Stemulate™ negatively influenced the re-differentiation potential of chondrocytes in 3D pellet culture

To investigate whether the removal of Stemulate™ at the end of chondrocyte monolayer expansion resulted in delayed ECM production in 3D pellet culture, a 28 d time course was performed. 3D pellets were assessed for cell number (Fig. 4), total sGAG (Fig. 5), total collagen (Fig. 6) and gene expression by q-PCR (Fig. 7).

For both supplement types, cell number (Fig. 4) remained consistent throughout the time course ($p = 0.84$). This was expected since the 3D pellet conformation should enable the chondrocytes to recover their morphology and prevent further
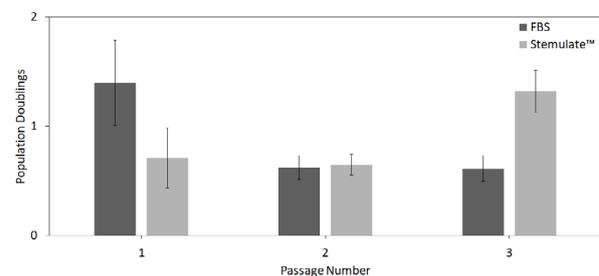

Fig. 1. Absolute population doublings of chondrocytes expanded in FBS and Stemulate™ sera at each of the three passages. Data are presented as mean ± SEM ($n = 5$).
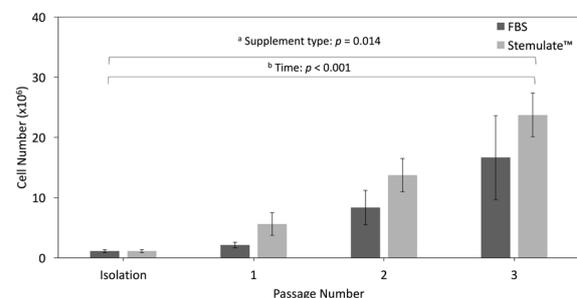

Fig. 2. Cell number during monolayer expansion of chondrocytes in FBS- and Stemulate™-supplemented sera. Cell number was calculated from cell counts during chondrocyte (seeded at an equal cell density) isolation and across three passages. Data are presented as mean ± SEM ($n = 5$).
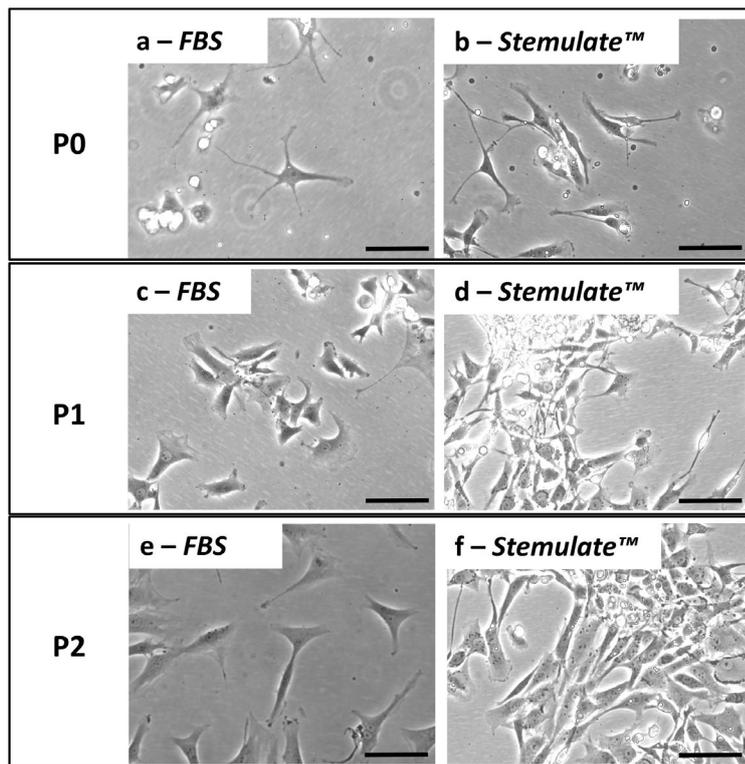
Fig. **3.** Representative images of chondrocytes expanded in culture media supplemented with 10 % (**a**,**c**,**e**) FBS and (**b**,**d**,**f**) Stemulate™ at passage 0-2 (P 0-P 2). Images were taken at day 4 of each passage to allow the chondrocytes to adhere. All images were taken using the Nikon TS100 light microscope and ×20 magnification. Images were acquired using the IPLab 3.2.6 software. Scale bars: 10 μm.

proliferation. Total sGAG (Fig. 5) and total collagen (Fig. 6) production were normalised to cell number. Chondrocytes expanded in both supplement types demonstrated a steady and significant increase in sGAG production across all time points ($p < 0.001$) (Fig. 5). sGAG concentration was higher in FBS as compared to Stemulate™ across all time points ($p = 0.009$), as determined by multi-level modelling. A significant sharp increase in sGAG concentration at day 14 was observed for FBS-expanded chondrocytes ($p < 0.001$), with a slight levelling off in sGAG production at day 28 for both supplements. No evidence of variability among individual patients at any time point was detected ($p = 0.60$) (Fig. 5).

Collagen production demonstrated a significant interaction between treatment and time ($p = 0.001$) (Fig. 6). Both supplement types showed a similar pattern of relatively steady levels until a sharp increase between day 21 and 28, with levels being significantly higher in FBS as compared to Stemulate™ ($p = 0.001$). Again, there was no evidence of variability among individual patients at any time point ($p = 0.26$) (Fig. 6).

RT-qPCR was performed at day 0, 21 and 28; relative gene expression profiles were determined by the comparative C_T method (Schmittgen and Livak, 2008) and normalised to the cell number. Expression of *COL1A1*, *COL10A1* or *ALK1* was not detected in either supplement type. For the key chondrogenic markers *ACAN*, *COL2A1* and *SOX9* there were striking differences between gene expression profiles in each supplement. *ACAN* expression increased over the time course in both supplements ($p < 0.001$ for time), but was significantly higher in FBS- than Stemulate™-expanded pellets at day 0 ($p < 0.001$)

and day 21 ($p = 0.013$). *COL2A1* expression was significantly higher in FBS- than in Stemulate™-expanded pellets at day 0 ($p = 0.033$) and day 21 ($p = 0.034$). FBS-expanded pellets demonstrated a significantly higher expression of *SOX9* at day 21 ($p = 0.012$) and day 28 ($p = 0.002$) when compared with Stemulate™-expanded pellets. There was no evidence of variability among patients for *ACAN* ($p = 0.48$) and *COL2A1* expression ($p = 0.33$) or for a general change in *COL2A1* ($p = 0.20$) and S*OX9* expression ($p = 0.15$) over time (Fig. 7).

FBS improved the quality of the ECM produced in 3D pellet culture
Frozen chondrogenic pellets were sectioned (7 μm) and stained with haematoxylin and eosin (H&E) or toluidine blue to visualise general morphology and proteoglycan distribution, respectively (Fig. 8). At day 7, chondrocytes expanded in Stemulate™ appeared to form less stable and more fragile pellets when compared with chondrocytes expanded in FBS. Morphological differences seemed apparent in the cells occupying the periphery of the pellets. These cells appeared to be more fibroblastic in pellets created from the Stemulate™-expanded chondrocytes as compared to those created from FBS-expanded chondrocytes (Fig. 8). In both supplement types, there was a trend for the staining intensity of the chondrogenic pellets to increase over time. Pellets from FBS-expanded chondrocytes demonstrated an increase in metachromasia. This increase was quantitated using ImageJ software (Fig. 9), to confirm that, at day 21, FBS-expanded chondrocytes had a significantly more intense staining as compared to Stemulate™-expanded chondrocytes ($p = 0.045$).
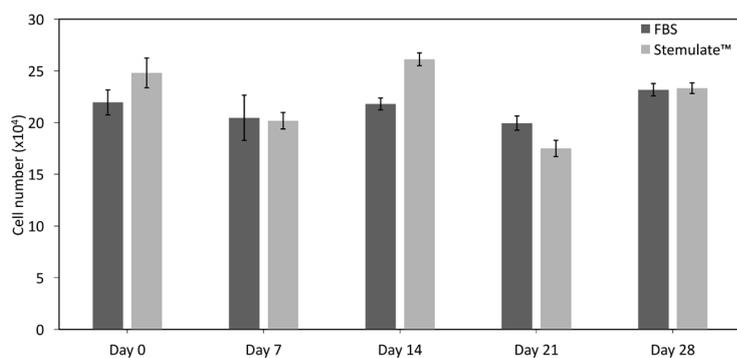
Fig. **4.** Cell number in chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplemented sera over a 28 d time course. Data are presented as mean ± SEM for day 0-21 (n = 5) and up to day 28 (n = 3).
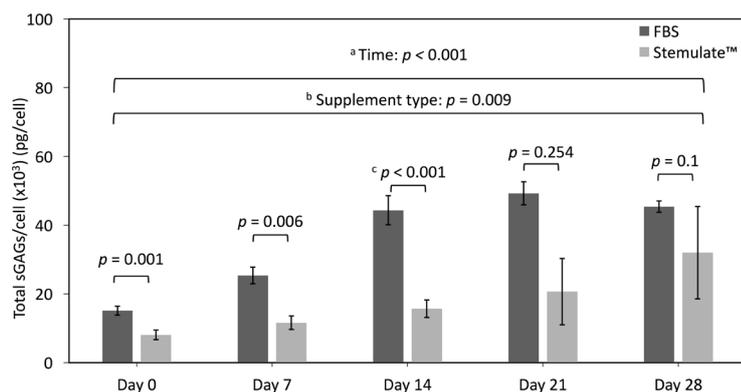

Fig. **5.** sGAG production per cell in chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplmented sera over a 28 d time course. Data are presented as mean ± SEM for day 0-21 (n = 5) and up to day 28 (n = 3). [a] denotes statistical significance for time. [b] denotes statistical significance for supplement type. [c] denotes statistical significance at day 14.

Again, there was no evidence of variability among patients in staining intensity (p = 0.48).

Discussion

This study focused on two sera supplements and their effects on human chondrocyte proliferation and the chondrocytes' subsequent recovery using standard chondrogenic differentiation protocols. To our knowledge, this is the only study providing a detailed analysis of ECM production from 5 individuals. However, it was not possible to compare AS among donors because the existing ethically approved protocol does not allow taking a sufficient volume of blood to provide sera for monolayer *in vitro* expansion to the end of passage 2, representing a limitation of the study. Another limitation was that only chondrocytes obtained from total knee replacement were evaluated. Therefore, caution must be used when interpreting these data as compared with chondrocytes sourced from patients undergoing ACI, as the patient populations for these treatments differ (for example, patients undergoing ACI tend to be younger than those undergoing joint replacement surgery). Despite these limitations, four key points were demonstrated. First, Stemulate™ increased chondrocyte proliferation when compared to FBS. This result was not surprising since several studies report that HPL increases cell proliferation in many cell types (Crespo-Diaz *et al.*, 2011; Hemeda *et al.*, 2014; Hildner *et al.*, 2015; Luttenberger *et al.*, 2000; Mojica-Henshaw *et al.*, 2013). Second, monolayer

expansion of human chondrocytes in Stemulate™ negatively influenced the stability of the 3D pellets as compared to expansion in FBS: these chondrogenic pellets disintegrated easily when sectioned, slightly losing the pellet form – especially in the cells occupying the periphery, which appeared to be more fibroblastic. Third, total sGAG synthesis by Stemulate™-expanded chondrocytes lagged behind the FBS-expanded ones. Fourth, expansion in Stemulate™ negatively influenced chondrogenic gene expression in 3D pellets as compared to FBS.

There is clear evidence to confirm that 5-10 % (v/v) HPL is more effective in supporting monolayer expansion of human MSCs than both FBS and AS at the same concentration range (Bieback *et al.*, 2009; Juhl *et al.*, 2016; Trojahn Kølle *et al.*, 2013). Further, both Bieback *et al.* (2009) and Juhl *et al.* (2016) report that HPL supported long term monolayer expansion whilst still maintaining the MSCs phenotype and their differentiation potential. Given these positive data, herein it was established that Stemulate™ was better than FBS at increasing chondrocyte proliferation in monolayer culture. No published studies have used Stemulate™ for chondrocyte expansion, but increased proliferation is shown when using derivatives of HPL. Spreafico *et al.* (2009) compare the effects of platelet rich plasma (PRP), platelet poor plasma (PPP) and FBS on human chondrocytes over a 20 d cell proliferation time course, with PRP resulting the most stimulatory in terms of chondrocyte proliferation. Hildner *et al.* (2015) report that 5 %-10 % HPL significantly increase human chondrocyte proliferation as compared to FBS. From
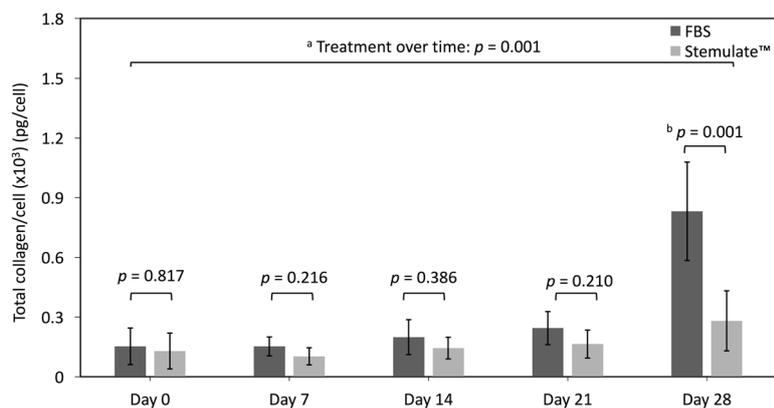
Fig. 6. Total collagen production per cell in chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplemented sera over a 28 d time course. Data are presented as mean ± SEM for day 0-21 ($n = 5$) and up to day 28 ($n = 3$). [a] denotes statistical significance for time. [b] denotes statistical significance at day 28.
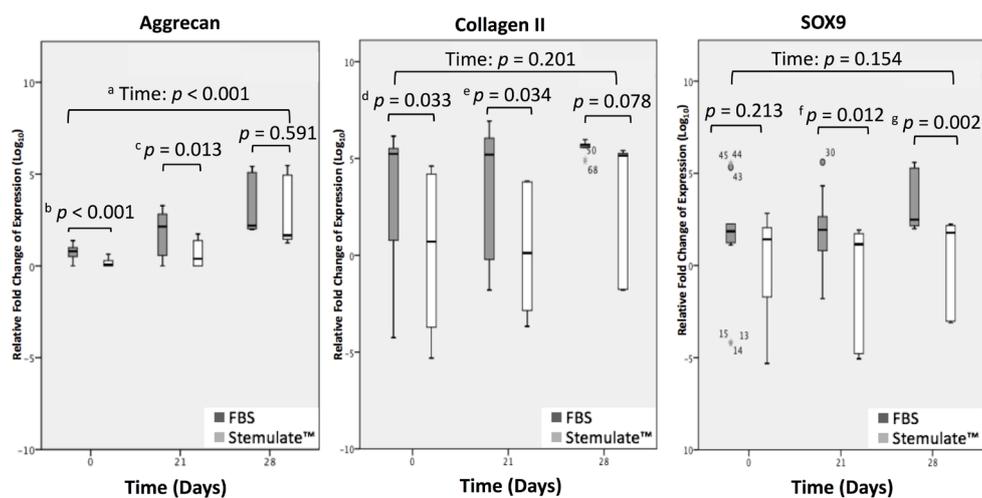

Fig. 7. Box and whisker plot of gene expression profiles of the chondrogenic pellets created from chondrocytes expanded in FBS- and Stemulate™-supplemented sera. Data are expressed relative to the reference genes, obtained from 5 patients using 3 experimental pellet replicates per patient, and determined by using the comparative C_T method. [a] denotes statistical significance for time. [b] denotes statistical significance for aggrecan expression at day 0. [c] denotes statistical significance for aggrecan expression between supplement types at day 21. [d] denotes statistical significance for collagen II expression between supplement types at day 0. [e] denotes statistical significance for collagen II expression between supplement types at day 21. [f] denotes statistical significance for *SOX9* expression between supplement types at day 21. [g] denotes statistical significance for *SOX9* expression between supplement types at day 28.

these studies and the current, it is evident that HPL increases the proliferation of human chondrocytes in monolayer culture.

In the current study, no morphological differences were observed in human chondrocytes that were monolayer-expanded in Stemulate™ or FBS. No other published study has investigated morphological differences between human chondrocytes expanded in HPL or FBS. However, Trojahn-Kølle *et al.* (2013) report morphological differences in monolayer-expanded MSCs, appearing smaller and less spindle-like when cultured in HPL as compared with FBS. In the current study, 3D pellets from human chondrocytes expanded in monolayer in Stemulate™ created more fragile, less compact pellets as compared to those expanded in FBS, as seen by qualitative histological assessment. These chondrogenic pellets disintegrated easily, losing

their pellet form, when prepared for histological analysis, resulting in a more difficult sectioning of the Stemulate™-expanded pellets at day 7. This was not assessed quantitatively but was a consistent observation throughout the study.

Very few studies use HPL with human chondrocytes and even fewer investigate sGAG production in 3D pellets. Following a published 3D pellet chondrogenic differentiation protocol (Johnstone *et al.*, 1998; Pittenger, 1999), it was demonstrated that FBS-expanded human chondrocytes generated significantly more sGAG than Stemulate™-expanded human chondrocytes across a 4-week time course. These results were opposite to the findings of Hildner *et al.* (2015), who report that HPL-expanded chondrocytes produce more sGAG than FBS-expanded chondrocytes in micromass pellets created from the same chondrogenic differentiation

protocol used in this study, over a 5-week period. After a 5-week culture period, a more intense sGAG staining is observed in HPL-expanded human chondrogenic pellets, compared to FBS pellets (Hildner *et al.*, 2015). These differences could be due to the supplement types, since Stemulate™ was used in the current study, whereas Hildner *et al.* (2015) use a HPL generated in their own laboratory from PRP obtained from 36 expired thrombocyte concentrates produced at a transfusion blood centre. 5 batches of HPL are characterised by Quantikine ELISA for these experiments, but no further details on the characterisation are given (Hildner *et al.*, 2015). Furthermore, in the current study, the 3D pellets were cultured in chondrogenic medium without HPL, whereas Hildner *et al.* (2015) add HPL into 3D pellet cultures.

In contrast to sGAG, total collagen production levels were found to be quite low throughout the 3D pellet culture 4-week time course. Despite this, collagen production by Stemulate™-expanded human chondrocytes appeared to lag behind FBS-expanded human chondrocytes. Gaissmaier *et al.* (2005) also observe that HPL-expanded chondrocytes seeded into 3D alginate beads produce low levels of collagen over a shorter time course of 14 d.

In the 3D pellet cultures, genes that determine chondrocyte differentiation state were investigated. De-differentiation was investigated through the relative expression of *COL1A1* (Benya *et al.*, 1978; Brew *et al.*, 2010) and hypertrophy through the relative expression of *COL10A1* (Caron *et al.*, 2012; Dell'Accio *et al.*, 2001) and *ALK1* (Blaney Davidson *et al.*, 2009; Dell'Accio *et al.*, 2001). *ALK1* is up-regulated during monolayer expansion, due to a loss of *in vivo* cartilage formation potential, and is associated with irreversible chondrocyte de-differentiation (Blaney Davidson *et al.*, 2007; Blaney Davidson *et al.*, 2009; Dell'Accio *et al.*, 2001). None of these genes were detected in 3D pellets derived from either Stemulate™- or FBS-expanded human chondrocytes, suggesting that the human chondrocytes had not undergone de-differentiation or hypertrophy. In addition, chondrogenic capacity was assessed through the relative expression of *COL2A1*, *ACAN* and *SOX9*. *COL2A1* and *ACAN* are necessary for ECM formation and the transcription factor *SOX9* regulates chondrocyte proliferation, chondrogenesis and transition to a non-hypertrophic state. Expression of *COL2A1* and *ACAN* declines following prolonged monolayer culture, particularly after passage 4 (Schulze-Tanzil *et al.*, 2002). For both these ECM markers, an increase was found across the 4-week time course. However, a much larger increase in relative gene expression was expected, especially by day 28, as this is found in similar published studies (Hildner *et al.*, 2015). An explanation could be that nutrient saturation was approached
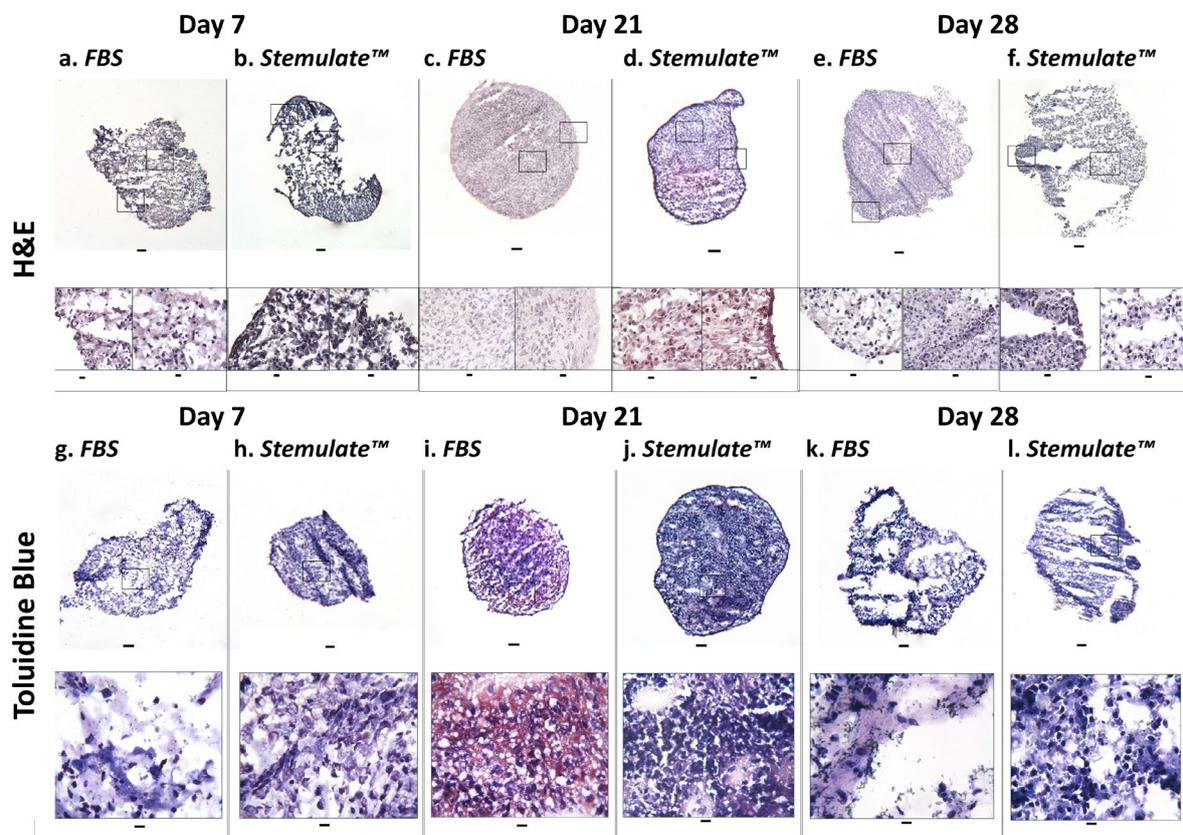

Fig. 8. Representative histological images from the centre of the FBS- and Stemulate™-expanded chondrogenic pellets across the 28 d time course. (**a**-**f**) Pellets stained with haematoxylin and eosin (H&E) to assess overall morphology. (**g**-**l**) Pellets stained with toluidine blue to assess proteoglycan content. Insert images are higher-powered images of the regions marked with a solid line. Scale bars: 100 μm.
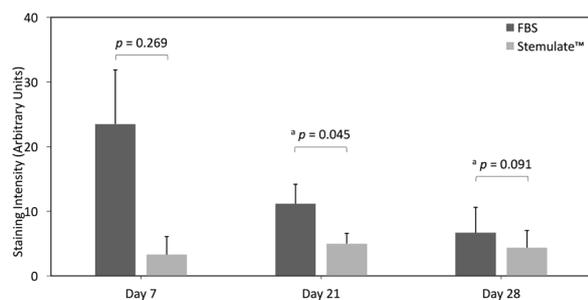
Fig. 9. Semi-quantification of toluidine-blue-stained chondrogenic pellets, demonstrating an increase in metachromasia, determined by integrated density analysis using ImageJ imaging software (version 24). Data are presented as mean ± SEM for day 0-21 ($n = 5$) and up to day 28 ($n = 3$). [a] denotes statistical significance for staining intensity between supplement types at day 21.

before day 28. Overall, *SOX9* gene expression was more striking in FBS 3D pellets when compared to Stemulate™, indicating that the FBS-expanded human chondrocytes recovered chondrogenic potential more quickly. Taken together, these findings suggest that Stemulate™ caused a delay in the recovery of chondrogenic phenotype following monolayer expansion. It would have been interesting to perform q-PCR on the monolayer chondrocytes prior to seeding into pellets and to compare the relative gene expression of the chondrocytes before and during pellet culture. This would have determined how the relative gene expression levels would have changed following chondrogenic differentiation. However, the chondrocytes used were primary isolated chondrocytes and monolayer-expanded up to passage 2, which limited cell number and prohibited the use of q-PCR analysis on the monolayer chondrocytes.

The multilevel modelling analyses performed allowed the comparison of the treatments over the time course and the exploration of the impact of patient variability (Hox, 2010; Vaughn, 2008). One of the limitations of the study was that cartilage was obtained from joints that were potentially osteoarthritic. OA is classified as a whole-joint disease and, although the cartilage was taken from macroscopically normal areas, chondrocytes could still have had a sub-chondrogenic phenotype upon differentiation. A previous study shows that osteoarthritic chondrocytes express higher levels of *ALK1* (Blaney Davidson *et al.*, 2009), but *ALK1* expression was not detected in the monolayer-expanded chondrocytes. Patient variability was expected, but no statistical evidence was found for a patient-specific influence on any of the measured outcomes. Clearly more work needs to be done, but the current work demonstrated that although Stemulate™ led to rapid proliferation, it affected the re-differentiation potential of human chondrocytes. These findings demonstrated that

Stemulate™ induced a sub-chondrogenic phenotype upon differentiation or enhanced monolayer-induced chondrocyte de-differentiation and reduced chondrogenic capacity. Therefore, it should be used with caution when expanding chondrocytes for clinical use.

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Editor's note: Replies to questions raised by reviewers were incorporated into the text of the paper, so there is no Discussion with Reviewers section.

The Scientific Editor responsible for this paper was Martin Stoddart.