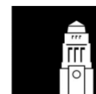

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1 # Optimising proliferation and migration of mesenchymal stem cells
2 # using platelet products: A rational approach to bone regeneration

3

4 MSC OPTIMISATION USING PLATELET PRODUCTS

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20 **Author Contributions Statement:**
21 All authors contributed extensively to the work presented in this paper. PVG, EJ, GT, SR,
22 and KM were responsible for the study conception and design. KM generated the platelet
23 products and performed the platelet characterisation, proliferation, migration and CFU-F
24 experimental work and analysis. JE conducted the flow cytometry experiments and analysis
25 and HO conducted the tri-lineage characterisation experiments and analysis. PVG was
26 responsible for patient recruitment and sample collection. Data analysis and interpretation
27 were performed by KM, EJ, JE and HO. Manuscript preparation and writing was completed
28 by KM and EJ with additional critical review by PVG, JE, HO, GT and SR.

29 All authors have read and approved the final submitted manuscript

30

Abstract:

This study investigates how mesenchymal stem cell's (MSCs) proliferation and migration abilities are influenced by various platelet products (PP). Donor-matched, clinical- and control laboratory-standard PPs were generated and assessed based on their platelet and leukocyte concentrations. Bone marrow derived MSCs were exposed to these PP to quantify their effect on *in vitro* MSC proliferation and migration. An adapted colony forming unit fibroblast (CFU-F) assay was carried out on bone marrow aspirate using clinical-standard PP-loaded electrospun poly(ε-caprolactone) (PCL) membrane to mimic future clinical applications to contain bone defects. Clinical-standard PP had lower platelet (2.5 fold, $P < 0.0001$) and higher leukocyte (14.1 fold, $P < 0.0001$) concentrations compared to laboratory-standard PP. It induced suboptimal MSC proliferation compared to laboratory-standard PP and fetal calf serum (FCS). All PP induced significantly more MSC migration than FCS up to 24 hours. The removal of leukocytes from PP had no effect on MSC proliferation or migration. The PP-loaded membranes successfully supported MSC colony formation. This study indicates that platelet concentrations in PP impact MSC proliferation more than the presence of leukocytes, whilst MSC migration in response to PP is not influenced by platelet or leukocyte numbers. Clinical-standard PP could be applied alongside manufactured membranes in the future treatment of bone reconstruction.

Key words: Stem cells; platelet products; bone regeneration; fracture repair

Introduction:

Fracture nonunion persists as a prevalent complication, with the incidence in long bones reported to range between 5-10% [1, 2]. Its etiopathogenesis remains multifactorial with a recent review identifying over 20 factors to be implicated in this process [3]. To address impaired fracture healing, Giannoudis PV et al. proposed the diamond conceptual framework for bone repair highlighting that, for a successful healing response both mechanical stability

and biological factors must be present [4]. For biological stimulation, while autologous bone graft remains the gold standard, recently, other forms have gained popularity including bone marrow aspirates (BMA) and/or growth factors [5, 6]. Since their introduction to the clinical setting, bone morphogenetic proteins (BMPs) have been extensively used for the treatment of nonunion fractures [7]. There are numerous publications reporting on the clinical results of BMP-2 and BMP-7 [8-10]. However, since the withdrawal of BMP-7 from the market and the license limitation of BMP-2 to be used for the management of open tibial fractures, other inductive molecules gained popularity such as demineralised bone matrix (DBM) [11], teriparatide [12] and platelet products (PP) [13, 14].

PPs are highly enriched sources of autologous growth factors and cytokines that act as biological stimulants to accelerate osteogenesis and bone repair [15, 16]. PPs are usually applied as platelet rich plasma (PRP), where the cells remain intact, or more recently, as platelet lysate (PL) where only the growth-factor containing plasma is used [17]. Both forms have been found to be highly effective in the treatment of orthopaedic trauma for decades [18], and more recently for the treatment of fracture nonunion [19]. Several studies into PP loaded membranes are already proving to be successful at promoting regeneration via the delivery of growth factors in cartilage and bone repair [20, 21].When working with PP loaded membranes, maximising bone marrow derived MSC (BM-MSC) proliferation and migration is key. By ensuring these functions are enhanced, more viable cells are available to differentiate, as well as inducing more resident BM-MSC homing to the site of injury. Whilst some studies found that the presence of leukocytes in PP are advantageous due to their antimicrobial properties[22] and high concentration of cytokines involved in bone repair such as VEGF [23, 24], there are also concerns related to leukocytes' effect of inducing excessive inflammatory and necrotic pathways from the surrounding tissue [25, 26]. Collectively, this provides a strong case for their depletion.

81 This study investigates the *in vitro* effect of platelets and leukocytes on the proliferation and
82 migration of MSCs from BMA by studying a range of PP compositions including a clinical-
83 standard PRP (CPRP). A laboratory-standard PRP made using a two-spin centrifugation
84 protocol to enrich platelets but deplete leukocytes was also used as control [18, 27].

85 To investigate the specific importance of leukocytes, PRP was processed further by syringe-
86 filtration to remove leukocytes and produce a pure filtered platelet rich product (fPRP). These
87 products were then lysed to ensure product consistency over the course of *in vitro* assays;
88 thereafter referred to as clinical-standard platelet lysate (CPL), platelet lysate (PL) and
89 filtered PL (fPL). These PLs were then evaluated *in vitro* utilising primary BM-MSCs,
90 including autologous BM-MSC-PP combinations, as well as loading onto a degradable
91 electrospun membrane to achieve sustained localised delivery in a colony forming units-
92 fibroblast (CFU-F) assay. The aim was to assess if high numbers of leukocytes would impede
93 BM-MSC proliferation and migration and whether their depletion from clinical PPs would be
94 desirable for bone regenerative approaches.

95 **<u>Methods:</u>**

96 This study was carried out in adherence with the Helsinki Declaration under ethics code
97 06/Q1206/127 following approval from the local National Health Service Research &
98 Development Department, Leeds East Research Ethics Committee to harvest these samples.
99 Written informed consent was obtained from each participant.

100 **<u>Sample collection and processing of CPRP, PRP and fPRP:</u>**

101 Whole blood was collected from 11 healthy volunteers (8 males and 3 females, 22-58 yrs) in
102 acid-citrate dextrose solution A (ACD-A). Each sample was divided and used to prepare
103 CPRP and PRP in a single-donor model. CPRP was made from whole blood using the
104 BioCUE^{TM} device (Zimmer Biomet) which was centrifuged at 1100g for 15 min. After

105 centrifugation, the device was agitated and the CPRP fraction was extracted using a syringe.
106 PRP was generated using a two-step centrifugation protocol [28] whereby the patients' whole
107 blood was incubated at room temperature for 1 hr before centrifugation at 400g for 10 mins.
108 The platelet-containing supernatant was extracted and centrifuged at 2700g for 10 mins
109 before resuspending the pellet in 1/5th of the residual supernatant.
110 fPRP was generated from PRP that was filtered through a white blood cell (WBC) syringe
111 filter (Acrodisc) which entraps leukocytes and allows platelets to pass through for collection.
112 The division of PRP for the production of fPRP meant both their final volumes were much
113 smaller than CPRP limiting the number of assays they could be used in. Platelet and
114 leukocyte concentrations of CPRP, PRP, fPRP and whole blood (1mL) were determined
115 using an automatic haematology analyser (Sysmex).

116 **Generating lysate products (CPL, PL and fPL):**

117 To generate the lysed products for *in vitro* assays, CPRP, PRP, and fPRP were processed
118 through three freeze-thaw cycles which involved freezing at -80°C followed by thawing at
119 37°C [29]. To remove contaminating cell debris, CPL, PL and fPL were centrifuged at 2700g
120 for 10 mins and the supernatants were extracted for cell stimulation.

121 **Sample collection and processing of BM-MSCs:**

122 Patients undergoing elective orthopaedic surgery with no underlying disease were recruited
123 (n=11, 6 males and 5 females, 17-69 yrs). 4 mL of BMA was collected from each donor's
124 anterior iliac crest and was treated with ammonium chloride to lyse red blood cells. The
125 remaining nucleated cells were plated into flasks at the cell seeding density of
126 $5x10^5$cells/cm^2. BM-derived adherent cells were cultured in MSC expansion media (Miltenyi
127 Biotec) for approximately two weeks until confluent.

128 **Characterising BM-MSCs:**

129 Cultured BM derived cells (passage two) were tested for the surface expression levels of
130 MSC markers as defined by the International Society for Cell Therapy (ISCT) [30]. Following
131 trypsinisation (Sigma Aldrich), cell cultures were stained with antibodies against positive
132 markers of MSCs (CD90, CD73 and CD105) and negative hematopoietic-lineage markers
133 (CD34, CD14, HLA-DR, CD19, CD45). All antibodies were used according to the
134 manufacturer's recommendation (Miltenyi Biotec). Isotype controls (BD, Bio-Rad) were
135 used to gate for positive expression. The data was acquired on an Attune flow cytometer
136 (ThermoFisher Scientific).

137 **Trilineage differentiation potential of BM-MSCs:**

138 BM derived cells were cultured up to passage four before being trypsinised and tested for
139 multipotentiality as previously described [31].Cells were seeded with AdipoDiff Media
140 (Miltenyi Biotec) or OsteoDiff Media (Miltenyi Biotec) for adipogenesis or osteogenesis,
141 respectively, with bi-weekly media changes. After 3 weeks, cell cultures were stained with oil
142 red solution (adipogenesis) [32] or Alizarin Red (osteogenesis). For chondrogenesis, cells were
143 added to Eppendorf tubes to create a pellet, resuspended in ChondroDIFF Media (Miltenyi
144 Biotec) and cultured for 21 days after which the pellets were stained with toluidine blue
145 (Sigma).

146 **Proliferation assay:**

147 The XTT assay was used to quantify BM-MSC proliferation in the presence of different PL
148 products. Activity of mitochondrial dehydrogenase, and therefore cell number per well, is
149 directly correlated to the amount of orange formazan formed, as monitored by the optical
150 density (OD) at 450nm. BM-MSCs were seeded in triplicate in a 96-well plate at a density of
151 500 cells/well with MSC expansion media and incubated for 24 hrs. The media was then
152 replaced with basal Dulbecco's Modified Eagle's Media (DMEM; Sigma-Aldrich)

153 supplemented with 10% (v/v) of CPL, PL, fPL or fetal calf serum (FCS) and incubated for
154 five days with a half media change on day three. Following exposure to the treatment media,
155 cell proliferation was assessed on day five using an XTT cell proliferation kit (Roche)
156 according to the manufacturer's instructions.

157 **Migration assay:**

158 The bottom wells contained basal DMEM (1% (v/v) Penicillin/Streptomycin and 2 I.U/mL
159 sodium heparin solution) supplemented with 10% (v/v) of CPL, PL, fPL or FCS. 10% was
160 the selected concentration due to its frequent use in the literature [33-35]. Migration was
161 analysed over 24 hrs using an IncuCyte® (Essen) to collect phase-contrast images of the
162 transwells as cells migrated along the chemotactic gradient. Images were captured every 30
163 min and a processing mask was created for each BM-MSC culture to account for donor
164 variation.

165 **CFU-F assay with CPL-loaded membrane:**

166 An adaptation of the CFU-F assay was used to quantify how a CPL-loaded membrane
167 affected the proliferation and migration capacity of uncultured BM-MSCs and to further
168 validate the results of the assays using cultured BM-MSCs. First, fresh BMA from three
169 donors underwent red blood cell lysis after which a total of 5 x 10^5 cells were plated in 15mL
170 expansion media to adhere over 24 hrs. After initial attachment and a PBS wash the media
171 was replaced with either 15 mL expansion media containing 2 I.U/mL sodium heparin
172 solution (control) or 15 mL DMEM media (no serum) containing 2 I.U/mL sodium heparin
173 solution (test). 1 cm^2 square sections, 400 µm thick, of UV-sterilised PCL that were made in-
174 house [36] were soaked in CPL for 15 mins until saturated before being placed in the centre of
175 the test dishes. The dishes were incubated for a further 19 days with half-media changes once
176 a week. After 21 days, the dishes were washed in PBS, fixed in 10% (v/v) formalin (Sigma)
177 and stained in 1% (w/v) methylene blue (Sigma). All colonies were imaged using a plate

178 scanner (Epson) at 1200 dpi. Colony area, integrated density and number per dish was
179 quantified using ImageJ whereby scanned images were converted to 8-bit greyscale and a
180 threshold mask was applied before particles were automatically analysed and measured.

181 **Results:**

182 **1) Characterising cellular content of PP:**

183 CPRP was generated using a BioCUE™ device whilst PRP was made following a previously
184 optimised protocol [18] and their cell populations were quantified using a haematology
185 analyser. Whilst both PP significantly enriched platelets compared to their whole blood
186 counterparts, PRP also had significantly higher numbers of platelets (15.9×10^5 PLT/µL)
187 (figure 1A) in comparison to CPRP (6.3×10^5 PLT/µL) as well as significantly lower
188 leukocyte numbers (1.8×10^3 LEUK/µL) compared to CPRP (20.6×10^3 LEUK/µL) (figure
189 1B). Despite the significant difference in final volumes (P=0.0078) (figure 1C), both products
190 had comparable average fold decrease in volumes (10.0 ± 0.0 and 7.9 ± 0.5 CPRP and PRP
191 respectively). All CPRP replicates underwent identical reductions in volume causing their
192 data points to overlap. The difference in the PP's cell populations is also visible in their
193 appearance as CPRP is opaque with additional red blood cell contamination (figure 1D)
194 whilst PRP had fewer contaminating red blood cells and was more translucent (figure 1E).
195 As previous studies have found that platelets and leukocytes aren't stable at room
196 temperature for the five days necessary for the proliferation and migration assays [37], the PP
197 were lysed after production to ensure consistency of samples. Lysis of the products changed
198 their nomenclature from CPRP and PRP to clinical platelet lysate (CPL) and platelet lysate
199 (PL).

200 **2) Characterising BM-MSCs:**

201 BMAs underwent red blood cell lysis, after which the remaining nucleated cells were plated
202 into flasks, three randomly selected cultures were tested for MSC characterisation according

203 to the ISCT's definition [38]. Cell surface marker expression was analysed using flow
204 cytometry which found cells were negative for CD14, CD19, CD34, CD45, HLA-DR and
205 positive for CD73, CD90 and CD105 (figure 2A) consistent with the criteria for identifying
206 MSCs. Their tri-lineage differentiation capacity was confirmed following three-week culture
207 in adipogenic, osteogenic and chondrogenic stimulation medias. A representative culture is
208 shown in figure 2 which was stained with oil red o solution to visualise lipid droplets (figure
209 2B), alizarin red staining to visualise calcium depositions (figure 2C), and finally toluidine
210 blue staining of the aggregated cell pellet indicative of glycosaminoglycan production. Nine
211 of these cultures were used for subsequent PL proliferation and migration tests, each PL
212 product was tested on at least three MSC cultures and the results were averaged.
213
214 **3) Platelet product composition's impact on BM-MSC proliferation:**
215 Cultured cells were exposed to 10% PL, CPL and control FCS containing media for five
216 days, after which the cells were treated with XTT reagent causing a colour change. BM-MSC
217 proliferation was expressed as OD normalised to FCS. BM-MSCs treated with PL were found
218 to proliferate significantly more than cells treated with CPL (P=0.0001) and equal to cells
219 treated with FCS (figure 3A). The same response was also seen from BM-MSCs tested with
220 autologous PL and CPL (figure 3B). To investigate whether CPL's suboptimal support of
221 BM-MSC proliferation was due to the high number of leukocytes, they were filtered out
222 using a leukocyte syringe filter. The filtered PL's (fPL) platelet numbers were not
223 significantly affected by the filtration process whilst leukocytes were shown to be
224 significantly depleted in fPL (figure 3C). Removal of the leukocytes was found to not
225 significantly improve proliferation as it was shown that both PL and fPL induced the same
226 BM-MSC proliferation as FCS (figure 3D).

227 Altogether, this data indicated that the leukocytes were not inhibitory for BM-MSC
228 proliferation and suggested that differing performances of PL and CPL observed were likely
229 due to the different numbers of platelets present.
230

231 **4) Platelet product composition's impact on BM-MSC migration:**

232 To compare the chemotactic potential of the different PPs, the IncuCyte® transwell assay
233 was used. Serum starved BM-MSCs were seeded on top of the transwells and exposed to
234 different PPs below. Over the course of 24 hours, the top and bottom of the transwells were
235 imaged continuously to track their migration. Representative images of the top of the
236 transwells are shown in figure 4A where non-migrated cells are shown in focus (purple
237 arrow), cells that have migrated through and identified by the software are highlighted in
238 green for processing (blue arrow) and the pores are identified with black arrows. The
239 migratory effects of PL and CPL were demonstrated as a time-course assay using one
240 representative culture (figure 4B) which showed that, both PL and CPL appear to induce far
241 more migration than 10% FCS. To enable statistical analysis, three independent PL
242 preparations were each tested on three BM-MSC cultures and found that both PL and CPL
243 induced significantly more migration than FCS at 12 hours (P= 0.0068 and P= 0.0434
244 respectively) and at 24 hours (P= 0.0006 and P= 0.0008) (figure 4C). Considering the higher
245 levels of leukocytes in CPL, these findings suggest that the presence of leukocyte-derived
246 proteins is not a detriment to BM-MSC migration and potentially contribute towards
247 improved migration.

248 To further investigate the effect of leukocyte-derived proteins on BM-MSC migration, PL
249 and fPL's chemotactic effects were investigated. A representative culture is shown in figure
250 4D which showed that, whilst the complete removal of leukocytes did appear to reduce BM-
251 MSC's migratory response this reduction was not statistically significant. In addition, PL and

252 fPL both induced far more migration than 10% FCS. BM-MSCs were found to be
253 significantly more migratory towards PL and fPL than 10%FCS at 12 hours (P= 0.0015 and
254 0.0198 respectively) and PL was also found to outperform 10% FCS at 24 hours (P= 0.0024)
255 (figure 4E). These findings suggest that whilst platelet and leukocyte numbers contribute
256 towards BM-MSC migration, it is likely that most of the cytokines that induce migration are
257 present in the plasma component of PP – and so any additional platelet or leukocytes make no
258 significant difference.

259 **5) Loaded membrane supports colony formation from native MSCs present in BMA:**

260 To more closely mimic clinical applications involving a PP-loaded biomaterial membrane as
261 a MSC homing and containment device, experiments were performed using clinically
262 approved PP. The aim of these experiments was to study if CPL released from a membrane
263 could support colony formation from rare BM-MSCs without their culture-amplification. In
264 standard CFU-F assays, single rare BM-MSCs give rise to individual colonies. The fresh
265 BM-MSCs used for this assay were grown in either standard MSC expansion media or basal
266 serum-free media with the addition of a CPL-loaded membrane (figure 5A). Colony
267 formation was observed in all test dishes, with similar morphology to control dishes
268 containing MSC expansion media (figure 5B). Furthermore, although there were trends for
269 higher colony numbers in MSC expansion media, and higher colony areas and densities in
270 CPL-loaded membrane dishes, the differences were not found to be statistically significant
271 (figure 5C). This indicated that CPL-loaded membranes were able to release growth factors
272 that induced colony formation and supported rare BM-MSC proliferation.

273 **Discussion:**

274 Whilst the clinical effectiveness of PP is generally accepted, due to the lack of quality
275 control, the variation in manufacturing, processing, delivery and its different applications

276 (e.g. rotator cuff repair and osteoarthritis [39, 40]), the 'optimal' composition is still hotly
277 debated depending on its specific clinical application.

278 The main issue with lack of standardisation is exemplified by one study which found that
279 PRP had no significant effect on bone healing [41], however, a closer look at the platelet
280 numbers used in the study found that some of the PRP tested had over 16-fold more platelets
281 than other PRP. Despite the vast differences in platelet numbers and omitted leukocyte
282 numbers, these products were all classified under the same umbrella term 'PRP'. This is
283 commonplace across the literature, whereby, the absence of standardised nomenclature,
284 quality control, as well as thorough analysis of cellular contents, makes it difficult to draw
285 clear conclusions. According to Delong et al.'s classification system [42] platelet number,
286 activation method and white blood cell number (PAW), CPRP is classified as P2-A (which
287 identifies the increase of platelets as 'moderate' and the leukocytes as 'enriched above
288 baseline') whilst PRP is classified as P3-B (which identifies the platelet concentration as
289 'highly enriched' above baseline and leukocytes depleted below baseline).

290 As well as variation in platelet concentrations, the lack of regulation also instils concern in
291 the scientific community over the use of highly concentrated leukocytes, their associated pro-
292 inflammatory cytokines (specifically TNF-α and IL-1β), and the risk that they could
293 counteract the platelet's beneficial effects and impede bone regeneration [26]. TNF-α and IL-
294 1β are known to induce inflammation causing a biphasic physiological response; whilst
295 inflammation is necessary for healing [43], in excess it is thought to activate the NFκB
296 pathway [26] inhibiting osteogenesis and promoting osteoclastogenesis [44]. However, more
297 recently, these proinflammatory cytokines have been linked to increased osteogenesis [45],
298 BM-MSC migration [46] and proliferation [47] indicative of the lack of consensus in the field.

299 This study addressed these concerns by better defining platelets and leukocyte's effects in the
300 two key physiological processes of bone regeneration: BM-MSC proliferation and BM-MSC
301 migration. With regards to proliferation, when compared to PL and FCS, CPL induced
302 significantly less BM-MSC proliferation than PL. This was also observed using donor-
303 matched BM-MSCs and PP proving that the response from the cells was not due to the
304 allogeneic nature of the PL. The cause of CPL's suboptimal ability to support BM-MSC
305 proliferation compared to PL is either due to its lower number of platelets or higher number
306 of leukocytes – both of which have been reported to reduce proliferation in the literature [26,
307 42, 48, 49]. To identify which cell type is the key player, leukocytes were filtered from PL whilst
308 platelet numbers were kept the same to produce fPL. Direct comparison of PL and fPL
309 showed that removing leukocytes did not improve proliferation. This suggests that in our
310 experimental conditions, the cytokines released from leukocytes neither enhanced nor
311 inhibited BM-MSC proliferation and that platelets are most likely responsible for releasing
312 the predominant growth factors involved in supporting BM-MSC proliferation.

313 With regards to BM-MSC migration, all three platelet products (PL, CPL and fPL) induced
314 significantly more migration than FCS, likely due to the greater concentrations of cytokines
315 such as VEGF and SDF-1 [50, 51]. Again, it was seen that removing leukocytes from PL did not
316 further enhance BM-MSC migration, but even seemed to reduce it. Whilst there is no
317 precedent for the use of platelet products in BM-MSC migration studies, the current data
318 supports previous literature of platelet products outperforming FCS [26, 34]. The methods used
319 are also unique in that, not only is the total migration shown, but also the increased rate of
320 cell migration towards the platelet products.

321 Based on these findings it could be proposed that generating high quality platelet products
322 should simply involve increasing platelet numbers as much as possible without regard for
323 leukocytes – this could be easily achieved by decreasing the final volume to yield a more

concentrated platelet product. However, the excessive enrichment of platelets faces the risk of paradoxically inhibiting cell proliferation, viability and migration [42]. A therapeutically effective range of platelet concentration is likely to be the case rather than a specific pure concentration. Our results indicate that platelet concentrations in the range of 6.3 -15.9 x10^5 PLTs/µL (with and without leukocytes) were effective in supporting BM-MSC proliferation and migration without adverse effects on their attachment or morphology.

Whilst several studies have already shown that PP increases the bone regeneration rate and prevention of non-union fractures in animal models [52, 53] and human subjects [14], as well as this work that aims to optimise PP's impact on BM-MSCs, it is also important to consider how PP should be delivered to the site of injury. To address this, a membrane was loaded with CPL and its discharge was found to support BM-MSC colony formation and BM-MSC proliferation. Due to the manufacturing process of PL and fPL (specifically the division of samples), low volumes prevented their loading onto a membrane for CFU-F analysis. In summary, this study supports the notion that the specific clinical application and desired outcome should be considered for defining best formulations of platelet products for bone regeneration. If cell proliferation is thought to be limiting regeneration, for example in elderly patients that have low numbers of autologous BM-MSCs [54], then CPL will be sub-optimal and PP with higher concentrations of platelets should be used. If however, the surgeon's priority is to induce BM-MSC migration to the site of injury, for example to attract BM-MSCs towards an unpopulated bone scaffold, then the current clinical standard CPL may be sufficient. As well, CPL was found to support colony formation when delivered using a membrane, with a trend of increased colony size and density than MSC expansion media; providing encouraging insight towards future delivery alternatives and streamlined surgeries.

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487 **Figure Legends:**

488 **Figure 1: Characterising platelet rich plasma products.** Using a haematology analyser, the
489 change in platelets (A), leukocytes (B) and final volume (C) from whole blood to CPRP

(n=11) and PRP (n=11) were quantified. The increase or decrease of each cell type indicates the change from whole blood (red) to the paired platelet product (black). A paired t-test was used to compare change in cell type before and after processing (**P<0.01, ***P<0.001 and ****P<0.0001) whilst a Mann-Whitney test was used to compare CPRP and PRP (#=P < 0.001, # #=P < 0.0001). Statistics could not be conducted on CPRP's volume analysis (C) due to matching differences between each sample. The photographs show the CPRP fraction product (D) and the PRP product (E).

Figure 1: Characterising bone marrow aspirate derived cultures. A) BM-MSC phenotyping using flow cytometry showing positive staining for markers of MSCs; CD105, CD90, CD73 and the absence of haem-lineage markers (Hemato); CD14, CD19, CD34, CD45 & HLA-DR. The bars show the mean positive percentage of BM cultured cells (n=3) with error bars representing standard deviation. Images of tri-lineage differentiation for adipogenesis (B), osteogenesis (C) and chondrogenesis (D), the black bars represent 500µm.

Figure 2: Platelet product composition's impact on BM-MSC proliferation. XTT assay quantifying BM-MSC proliferation following four day exposure to media containing 10% platelet products or FCS as control. Proliferation was represented as OD normalised to FCS. A) Cultured BM-MSCs were exposed to 10% PL, CPL and FCS. B) Cultured BM-MSCs were exposed to autologous 10% PL and CPL as well as 10% FCS. C) The change in platelets and leukocytes from whole blood to PRP and fPRP (compared using a paired t-test). D) Cultured BM-MSCs were exposed to 10% PL, fPL and FCS from three PL donors. One-way ANOVA test was used to test significance between the platelet products effects on proliferation (3A, B and D).(*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001). Except for 3B, all experiments were performed on a minimum of three different PP donors and three BM-MSC cultures. Error bars indicate variation between PP except for 3B where error bars indicate technical variation between replicates.

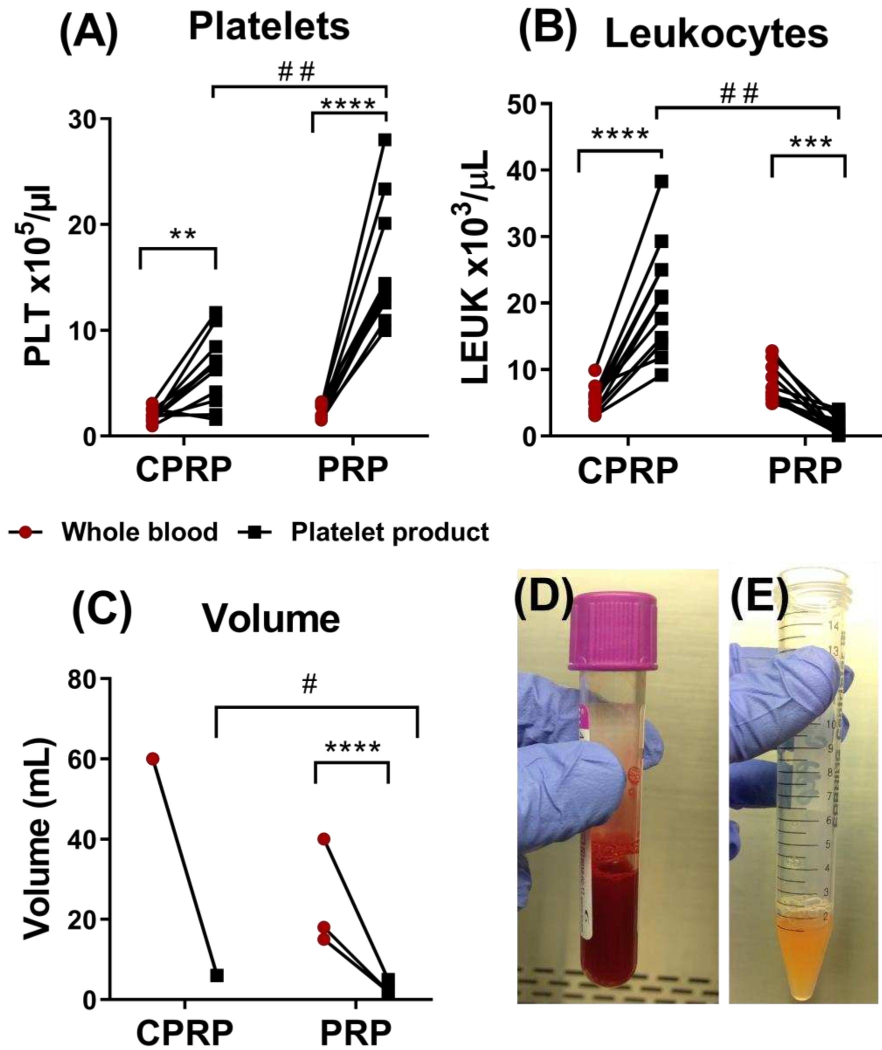
515 **Figure 3: Platelet product composition's impact on BM-MSC migration.** The IncuCyte®
516 transwell assay quantifies BM-MSCs moving towards a chemotactic gradient. A)
517 Representative images of wells of 0.5% FCS (top), 10% FCS (middle), and 10% PL (bottom).
518 Arrows indicate pores (black), static cells on the top of the transwell (purple) and cells that
519 have migrated (blue). The processing mask that quantifies the migrated cells is shown in
520 green. B) Representative time-course response of the cells from one BM-MSC culture that
521 have migrated through the transwell towards 10% PL, 10% CPL 10% FCS and 0.5% FCS.
522 Data is shown as the area of the bottom of the well occupied by cells. C) Average object area
523 of the underside of the transwell occupied by BM-MSCs and treated with 10% PL, 10% CPL,
524 and 10% FCS was normalised to 10% FCS. D) Representative time-course response of the
525 cells from one culture that have migrated through the transwell towards 10% PL, 10% fPL,
526 10% FCS and 0.5% FCS. E) Average object area of the underside of the transwell occupied
527 by BM-MSCs and treated with 10% PL, 10% fPL, and 10% FCS was normalised to 10%
528 FCS. A one-way ANOVA was carried out using the Kruskal-Wallis test for normality
529 (*$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$). Error bars indicate variation between
530 PL donors. Experiments in C and E were performed on a minimum of 3 different BM-MSC
531 cultures.

532 **Figure 4: CPL-loaded membrane supports cell proliferation in CFU-F assay.** A)
533 Representative CFU-F dishes of BM-MSCs grown in either standard expansion media (top
534 dish) or serum-free DMEM with the additional CPL-loaded membrane as a source of released
535 growth factors and cytokines (bottom dish). B) Representative individual colonies of cells
536 grown in expansion media (top) or CPL-loaded membrane and DMEM (bottom). Three BMA
537 donors were tested on a single CPL product. Images were collected using a photo scanner at
538 1200 dpi. C) Comparison of average colony area, density and total number between cells

539 treated with a CPL-loaded scaffold and expansion media (EM). An unpaired t-test found no

540 significant difference between CPL of EM.

541 **Figures:**



542

543 *Figure 5: Characterising platelet rich plasma products*.

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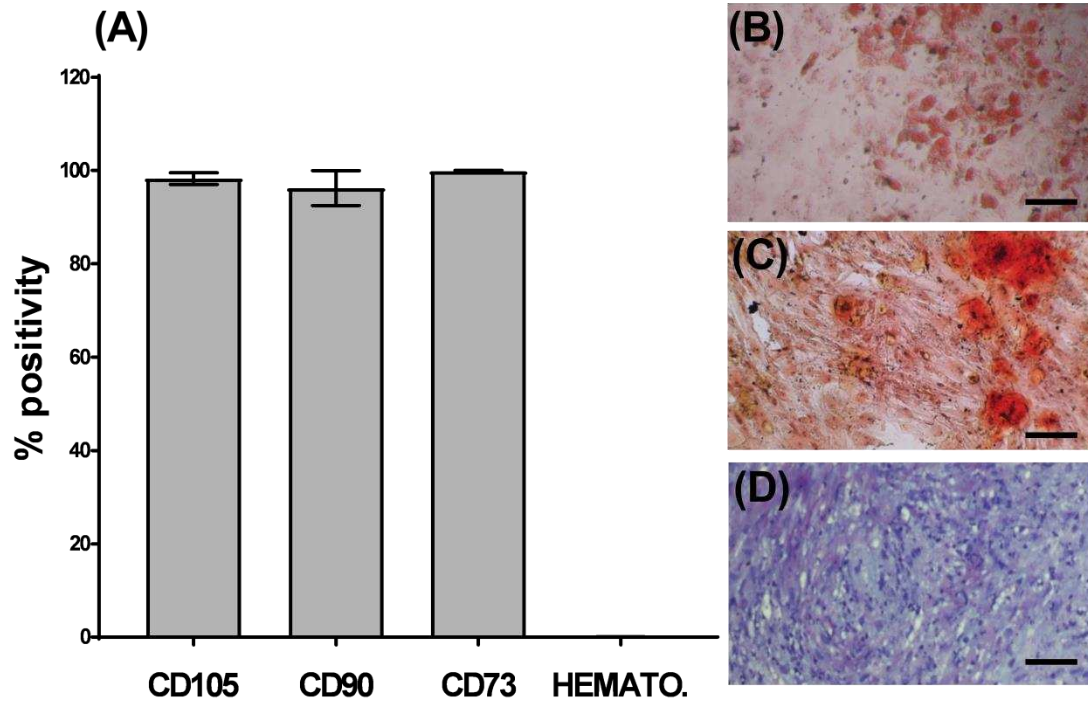

545 ***Figure 6: Characterising bone marrow aspirate derived cultures***.

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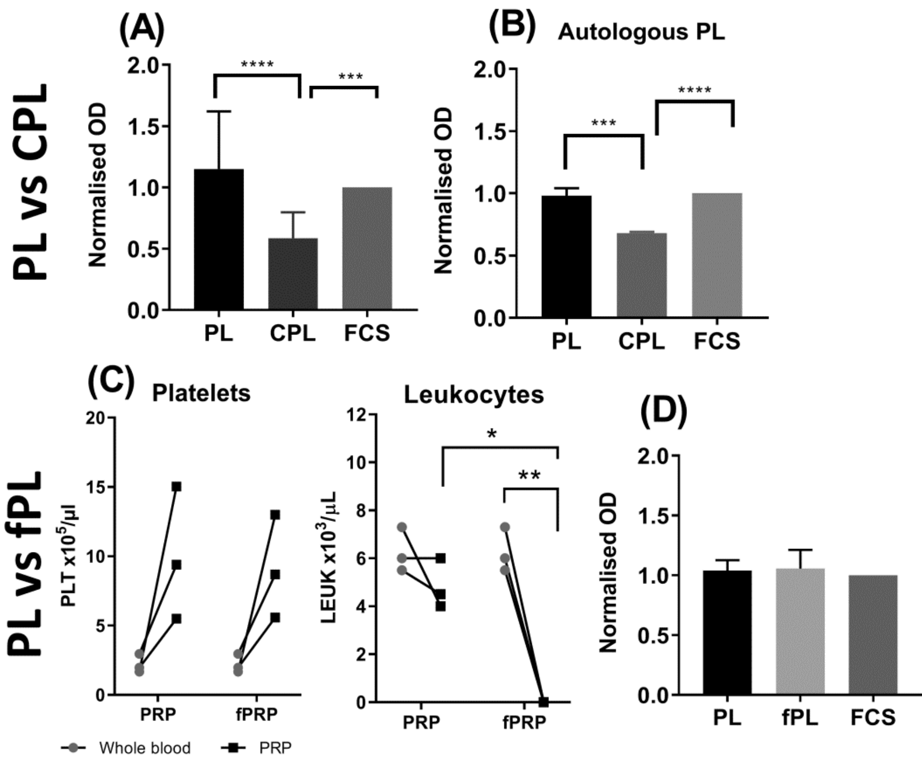

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549 ***Figure 7: Platelet product composition's impact on BM-MSC proliferation.***

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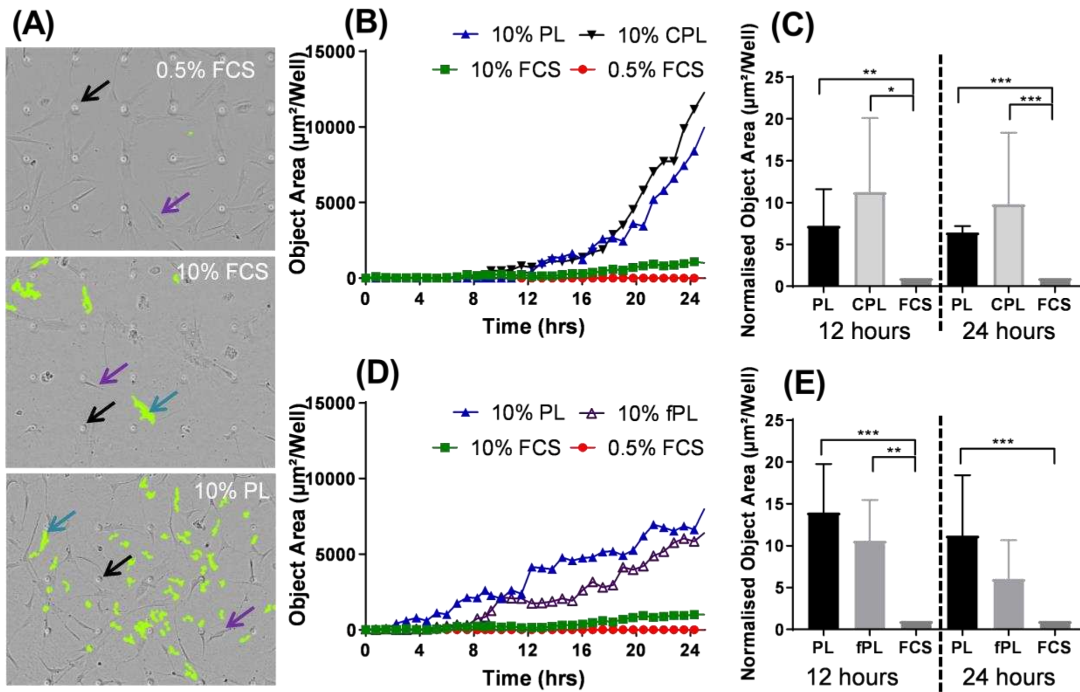

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552 ***Figure 8: Platelet product composition's impact on BM-MSC migration.***

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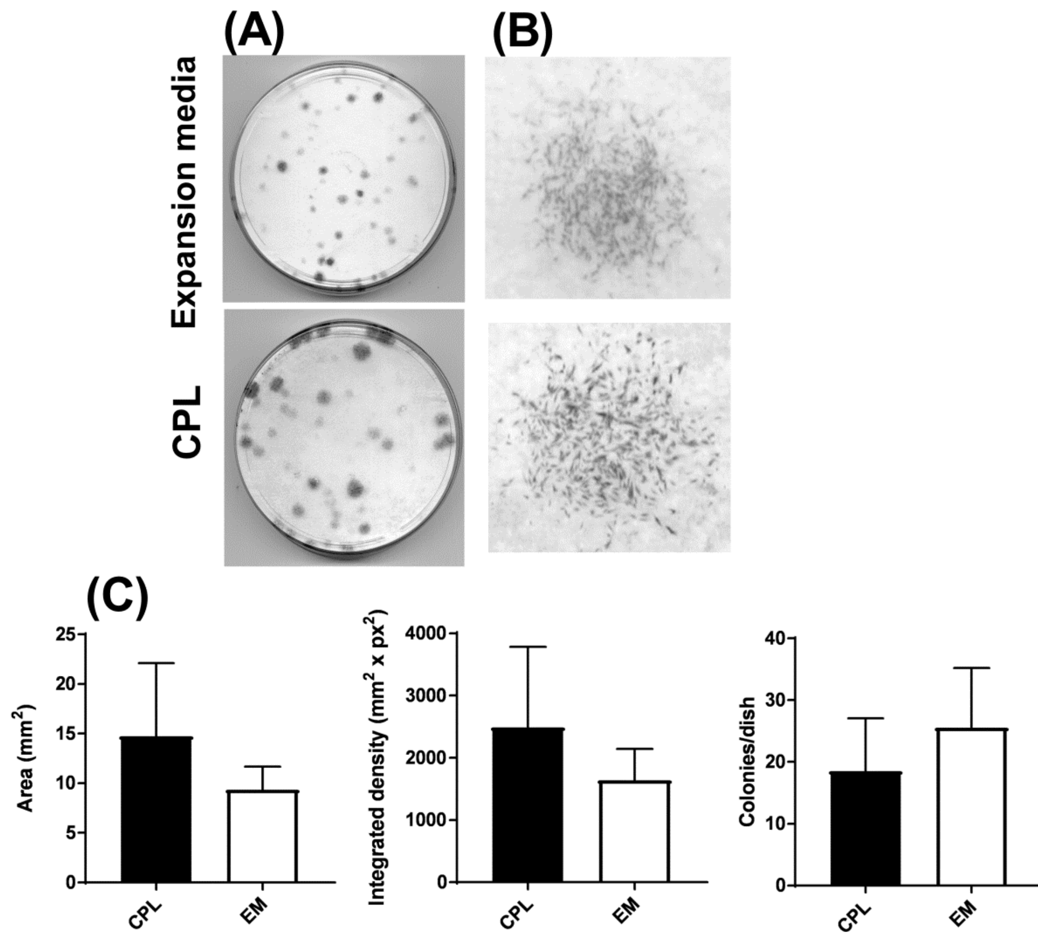

554

555 ***Figure 9: CPL-loaded membrane supports cell proliferation in CFU-F assay.***