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Effect of hypoglycemia on inflammatory responses and the response to low dose endotoxemia in humans

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Effect of Hypoglycemia on Inflammatory Responses and the Response to
2
Low Dose Endotoxemia in Humans

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19
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21
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24 and acknowledgements).
25

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40 Bayer. The other authors have no conflicts of interest to declare that relate to this topic of
41 research.

42 **Abbreviations:** ACCORD, Action to Control Cardiovascular Risk in Diabetes; AMI, acute
43 myocardial infarction; APC, allophycocyanin; AR1, autoregressive correlation structure;
44 AUC, area under the curve; BMI, body mass index; CCL2, C-C motif chemokine ligand 2;
45 CM, classical monocytes; CM-MPA, classical monocyte-platelet aggregates; CV,
46 cardiovascular; CXCL8, C-X-C motif chemokine ligand 8; CX_3CL1, CX_3C chemokine ligand
47 1; CX_3CR1, CX_3C chemokine receptor 1; FACS, fluorescence-activated cell sorting; FITC,
48 fluorescein isothiocyanate; IM, intermediate monocytes; IM-MPA. intermediate monocyte-
49 platelet aggregates; MPA, monocyte-platelet aggregates; NaCl, sodium chloride; NCM, non-

50 classical monocytes; NCM-MPA, non-classical monocyte-platelet aggregates; PBS,
51 phosphate-buffered saline; PE, phycoerythrin; STEMI, ST-elevation myocardial infarction;
52 WBC, white blood cell.

53 **Précis:** Using a novel *in vivo* human experimental model, we show that hypoglycemia primes
54 the innate immune system leading to a more profound inflammatory response to a subsequent
55 inflammatory stimulus.

56

57 # Abstract

58 **Context**

59 Hypoglycemia is emerging as a risk for cardiovascular events in diabetes. We hypothesized
60 that hypoglycemia activates the innate immune system, which is known to increase
61 cardiovascular risk.

62 **Objective**

63 To determine whether hypoglycemia modifies subsequent innate immune system responses.

64 **Design and Setting**

65 Single-blinded, prospective study of three independent parallel groups.

66 **Participants and Interventions**

67 Twenty-four healthy participants underwent either a hyperinsulinemic-hypoglycemic (2.5
68 mmol/l), euglycemic (6.0 mmol/l) or sham-saline clamp (n=8 for each group). Forty-eight
69 hours later, all participants received low-dose (0.3 ng/kg) intravenous endotoxin.

70 **Main outcome measures**

71 We studied *in-vivo* monocyte mobilization and monocyte-platelet interactions.

72

Results

Hypoglycemia increased total leucocytes (9.98±1.14 x10^9/l vs euglycemia: 4.38±0.53 x10^9/l; P<0.001 vs sham-saline: 4.76±0.36 x10^9/l; P<0.001) (mean±SEM), mobilized proinflammatory intermediate monocytes (42.20±7.52/µl vs euglycemia: 20.66±3.43/µl; P<0.01 vs sham-saline: 26.20±3.86/µl; P<0.05) and non-classical monocytes (36.16±4.66/µl vs euglycemia: 12.72±2.42/µl; P<0.001 vs sham-saline: 19.05±3.81/µl; P<0.001). Following hypoglycemia vs euglycemia, platelet aggregation to agonist (AUC) increased (73.87±7.30 vs 52.50±4.04; P<0.05) and formation of monocyte-platelet aggregates increased (96.05±14.51/µl vs 49.32±6.41/µl; P<0.05). Within monocyte subsets, hypoglycemia increased aggregation of intermediate monocytes (10.51±1.42/µl vs euglycemia: 4.19±1.08/µl; P<0.05 vs sham-saline: 3.81±1.42/µl; P<0.05) and non-classical monocytes (9.53±1.08/µl vs euglycemia: 2.86±0.72/µl; P<0.01 vs sham-saline: 3.08±1.01/µl; P<0.05) with platelets compared to controls. Hypoglycemia led to greater leucocyte mobilization in response to subsequent low-dose endotoxin challenge (10.96±0.97 vs euglycemia: 8.21±0.85 x10^9/l; P<0.05).

Conclusions

Hypoglycemia mobilizes monocytes, increases platelet reactivity, promotes interaction between platelets and proinflammatory monocytes, and potentiates the subsequent immune response to endotoxin. These changes may contribute towards increased cardiovascular risk observed in people with diabetes.

97 # Introduction

98 Hypoglycemia is associated with a greater propensity to adverse cardiovascular (CV)
99 outcomes in diabetes (1-3). To determine if such outcomes were dependent upon changes in
100 innate immune responses, we devised a novel model whereby subjects were challenged with
101 a hypoglycemic clamp, and then the durable effects on the innate immune system probed by
102 an *in vivo* endotoxin challenge 48 hours later.

103

104 Iatrogenic hypoglycemia remains a major barrier to effective treatment of insulin-treated
105 diabetes (4). The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial showed
106 that intensive glucose control, during which patients were exposed to significantly more
107 hypoglycemia (5), was associated with excess CV mortality. Despite the evidence confirming
108 an association between hypoglycemia and mortality, cause and effect has not been
109 established. Trial evidence suggests that the relationship is, at least in part, explained by
110 'confounding', i.e. that hypoglycemia identifies individuals with comorbidities who are both
111 vulnerable to hypoglycemia and more likely to die for other reasons (6). Nevertheless, a
112 recent large meta-analysis (7) has suggested that comorbidities alone are unlikely to explain
113 this relationship. Furthermore, there is a growing body of evidence highlighting a number of
114 mechanisms whereby hypoglycemia may lead to CV events (8, 9).

115

116 Hypoglycemia has proinflammatory consequences, including increases in levels of factor
117 VIII and von Willebrand factor and impaired fibrinolysis (10-12). In addition, hypoglycemia
118 has been shown to increase proinflammatory cytokines (12-14) and promote rises in the
119 levels of proatherogenic cell adhesion molecules (12). Repeated episodes of hypoglycemia
120 have also been reported to impair nitric oxide-mediated vasodilation (15).

121

122 Monocytes are phagocytes that are central to the etiology of atherosclerosis (16) and play a
123 role in precipitating acute CV events by promoting plaque destabilization and rupture (17).
124 The extent to which monocytosis and monocyte activation is modified by hypoglycemia
125 remains uncertain. Recent studies have also determined that monocytes can be classified into
126 3 distinct subsets, termed classical monocytes (CM: $CD14^{++}$ $CD16^{-}$, 'Mon1'), intermediate
127 monocytes (IM: $CD14^{++}$ $CD16^{+}$, 'Mon2'), and non-classical monocytes (NCM: $CD14^{+}$
128 $CD16^{++}$, 'Mon3') (18, 19). A number of observational studies indicate that IM may be
129 particularly proatherogenic. Elevated levels of IM are associated with adverse CV outcomes
130 (20-23), independently predict future CV events (22), and have been associated with coronary
131 plaque vulnerability in patients with angina (24). Elevated levels of CM may also
132 independently predict CV events (25).
133
134 Acute myocardial infarction (AMI) results in monocytosis, mediated by sympathetic nervous
135 system activation (26). In humans, $CD16^{+}$ monocytes selectively mobilize, in a
136 catecholamine-dependent fashion, following exercise (27). As epinephrine is the key counter-
137 regulatory hormone produced in response to hypoglycemia, we hypothesized that
138 hypoglycemia would also exert significant effects on monocytes. We further hypothesized
139 that we would see additional synergistic changes in monocyte and platelet activation, as
140 revealed by formation of monocyte-platelet aggregates, which are increased after AMI (20,
141 28). In large prospective studies, CV events did not appear to occur during the hypoglycemic
142 episode *per se* but there was an increased risk of events in the weeks and months following
143 the episode (29-31). We hypothesized, therefore, that acute hypoglycemia may prime the
144 innate immune system, leading to a more pronounced inflammatory response to a subsequent
145 inflammatory stimulus downstream from the initial episode of hypoglycemia. It is also
146 relevant to note that people with diabetes experience increased incidences of acute and

147 chronic infections that will further activate innate immunity. To reveal if hypoglycemia
148 modulated monocyte function in the human *in vivo*, we chose to combine a classical
149 hypoglycemic stimulus with a subsequent *in vivo* systemic stimulus of the innate immune
150 system. To achieve this, we combined hyperinsulinemic hypoglycemic-euglycemic and
151 sham-saline clamps with low-dose intravenous endotoxin challenge 48 hours later in healthy
152 participants. Endotoxin, otherwise known as Gram-negative bacterial lipopolysaccharide,
153 was employed as it induces a short-lived, sterile inflammation that is both safe and
154 reproducible (32).

155 # Materials and Methods

156 **Study Design and Participants**

157 This was a single-blinded, prospective study of three independent parallel groups
158 (hyperinsulinemic-hypoglycemia/euglycemia and sham-saline controls) conducted in a
159 random group order at the Clinical Research Facility, Northern General Hospital, Sheffield,
160 United Kingdom between January 2015 and April 2016. We therefore had three groups that
161 had euglycemia with insulin, hypoglycaemia with insulin, or saline. Each then received
162 endotoxin. Baseline values at the start of endotoxin administration were studied in all groups,
163 providing a set of data obtained prior to endotoxin. Twenty-four healthy participants without
164 diabetes were recruited from the University of Sheffield and Sheffield Teaching Hospitals
165 with written informed consent in accordance with a protocol approved by Yorkshire and the
166 Humber-Sheffield Research Ethics Committee (REC 14/YH/1264). All participants had a
167 HbA_{1C} < 6.5% (< 48 mmol/mol), measured using ion-exchange high-performance liquid
168 chromatography, and none had impaired glucose tolerance based on HbA_{1C} as judged by the
169 American Diabetes Association criteria (33, 34). Participants were in good health as
170 determined by a medical history, physical examination, vital signs and clinical laboratory test

171 results including full blood count and renal and liver function. Those with an intercurrent
172 illness in the previous 4 weeks were excluded. Participants taking beta-blockers, QT interval-
173 prolonging agents and anticoagulant, antiplatelet or antiinflammatory medications were also
174 excluded. Female participants were on secure contraception and also had negative urinary
175 pregnancy tests on the morning of the clamp and endotoxin studies.

176

177 **Clamp studies**

178 All participants attended at 0800h after an overnight fast and were blinded to their group
179 allocation. Participants were instructed to avoid caffeine, alcohol and vigorous exercise 24
180 hours before the study visit. An intravenous cannula was inserted into the antecubital fossa of
181 the non-dominant arm for insulin and dextrose infusion. A second intravenous cannula was
182 inserted into the antecubital fossa of the dominant arm for all blood measurements except
183 glucose. Following the application of a local anesthetic cream (EMLA, Astra-Zeneca,
184 Macclesfield, UK) to the dorsal hand or wrist of the non-dominant arm, a retrograde cannula
185 was inserted and the hand placed in a warming chamber (The Sheffield Hand Warmer,
186 Sheffield, UK) at 55°C to allow arterialisation of venous blood for glucose measurement. In
187 hypoglycemia and euglycemia study groups, a primed continuous insulin (Human Actrapid,
188 Novo Nordisk Pharmaceuticals LT, Crawley, UK) infusion was administered at a rate of 90
189 mU m^{-2} min^{-1} with total insulin exposure matched between groups. A 20% dextrose (Baxter,
190 Baxter Healthcare Ltd., Thetford, UK) variable rate infusion was administered
191 simultaneously and the rate adjusted according to arterialized whole blood glucose
192 concentrations measured every 5 minutes using a glucose oxidase method (Yellow Springs
193 Instrument 2300 STAT, Yellow Springs, Ohio, USA). Following a brief (30 minutes)
194 euglycemic phase in both groups, blood glucose was lowered to 2.5 mmol/l in the
195 hypoglycemia group and maintained for 60 minutes at this level. In the euglycemia group,

196 blood glucose was maintained at 6 mmol/l for 60 minutes. Participants in the sham-saline
197 group were investigated identically but did not receive insulin/dextrose infusions and instead
198 received a slow intravenous infusion of 0.9% sodium chloride (NaCl) (Baxter, Baxter
199 Healthcare Ltd., Thetford, UK) at a pre-determined fixed rate. Thus, participants in the sham-
200 saline group were under normoglycemic conditions allowing us to control for the effects of
201 insulin and dextrose. Blood was sampled at baseline and at 60 minutes. Members of staff
202 processing assays were blinded to glucose group allocation.
203
204 **Endotoxin challenge**
205 Endotoxin challenge is a safe and well-studied model of innate immune activation *in vivo*
206 (35). Forty-eight hours following the clamp, participants re-attended at 0800h having fasted
207 overnight and refrained from caffeine, alcohol and vigorous exercise since the clamp visit.
208 An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm for
209 administration of endotoxin and a second cannula inserted into the contralateral antecubital
210 fossa for blood sampling. All participants received 0.3 ng/kg *Escherichia coli* O:113
211 lipopolysaccharide (Clinical Centre Reference Endotoxin, National Institutes of Health,
212 Bethesda, Maryland, USA). Endotoxin powder was reconstituted in 1 ml of sterile 0.9% NaCl
213 to form a solution at a concentration of 1000 ng/ml, which was vortexed for 60 minutes. The
214 weight-adjusted dose of endotoxin was obtained from this solution, added to 5 ml of 0.9%
215 NaCl and administered as a slow bolus injection over 1 minute. An intravenous infusion of
216 500 ml of 0.9% NaCl (Baxter, Baxter Healthcare Ltd., Thetford, Norfolk, UK) then continued
217 for 4 hours following endotoxin to avoid hypotension. Venous blood was sampled at baseline
218 and 2, 4 and 6 hours following endotoxin. All laboratory measurements were performed by
219 staff blinded to glucose group allocation.
220

221 **Biochemical analysis**

222 To measure epinephrine, venous forearm blood was collected into chilled lithium heparin
223 tubes and centrifuged at 4°C, 1000*g* for 10 minutes. The resulting supernatant was stored at -
224 80°C until assayed by high-performance liquid chromatography. To determine insulin levels,
225 EDTA-anticoagulated blood was centrifuged at 3000*g* for 10 minutes, and free insulin levels
226 measured in the resulting plasma using an immunoassay (Roche Cobas, Roche Diagnostics,
227 Burgess Hill, West Sussex, UK). Venous blood was centrifuged at 3000*g* for 10 minutes and
228 the resulting serum used to measure cortisol and growth hormone using an
229 immunoradiometric assay (Roche Cobas, Roche Diagnostics, Burgess Hill, West Sussex,
230 UK). Sample collection for cortisol and growth hormone was controlled for time of day
231 across the three study groups.
232
233
234 **Cell counts and flow cytometry**

235 Total and differential white blood cell (WBC) and platelet counts in EDTA-anticoagulated
236 blood were determined using an automated clinical grade Sysmex cell counter (XN-9000,
237 Sysmex, Milton Keynes, UK). For the clamp visit, alternative WBC counting methodologies
238 were piloted for the first two subjects in each group but these were later deemed less accurate
239 than the Sysmex cell counter. Data shown below in Figures 2, 3 and 4a, 4c, 4d, 4e and 4f are
240 Sysmex data from n=6 in each study group. Flow cytometry was used to determine
241 monocyte-platelet aggregates (MPA): blood was collected into tubes containing trisodium
242 citrate dihydrate (3.13% w/v) and incubated in a heat block at 37°C for 10 minutes,
243 erythrocytes lysed using fluorescence-activated cell sorting (FACS) lyse solution (BD,
244 Oxford, UK) and stained with FITC-conjugated CD16 (BioLegend, London, UK), APC-
245 conjugated CD14 (BioLegend, London, UK) and PE-conjugated CD42a (BD, Oxford, UK) in

246 addition to matched isotype controls. Cells were fixed using FACS Fix (BD, Oxford, UK)
247 and analyzed using flow cytometry (Accuri C6 multi-colour flow cytometer, BD, Oxford,
248 UK) within a consistent time frame for all subjects. Monocytes were gated based on
249 morphology and CD14 expression. Neutrophils were gated on morphology and through
250 exclusion of monocytes. Monocyte-platelet aggregation was determined by measuring
251 monocyte mean fluorescence of the platelet-specific marker CD42a. To phenotype and
252 enumerate monocyte subsets, anticoagulated blood was stained with FITC-conjugated CD16
253 (BioLegend, London, UK), APC-conjugated CD14 (BioLegend, London, UK), PE-
254 conjugated CD66c (BD Biosciences, Oxford, UK), PE-Cy7-conjugated CD11b (BioLegend,
255 London, UK) and (PerCP)-eFluor® 710-conjugated CX3CR1 (eBioscience, Altrincham,
256 UK). Matched isotype controls and a 'fluorescence minus one' strategy optimized
257 compensation. Stained whole blood was lysed with FACS lyse as above, and the pellet
258 resuspended in PBS prior to fixation using 1% w/v formaldehyde. Samples were immediately
259 processed for analysis using flow cytometry (LSRII, BD, Oxford, UK). Monocytes were
260 gated based on morphological characteristics and through the exclusion of neutrophils using
261 CD66c. Monocyte subsets were identified based on relative expression of CD14 and CD16.
262 Flow cytometry data were analysed using Flow Jo (FlowJo, version 10, Ashland, Oregon,
263 USA).
264
265 **Platelet aggregation**
266 Platelet aggregation was measured using impedance aggregometry (Multiplate®, Verum
267 Diagnostica GmBH, Munich, Germany). Aliquots of 300μl saline and 300μl hirudin-
268 anticoagulated blood were added to the cuvette and incubated at 37 ^{o}C for 3 minutes. Twenty
269 microliters of adenosine diphosphate (ADP, at a final concentration of 6.45μM) was added as

270 agonist and the assay commenced. The area-under-the curve (AUC) was measured, which
271 represents the level of platelet aggregation.

272 **Statistical analysis**

273 Our pilot data indicated that a sample size of 7 participants per group would have 90% power
274 to detect a 50% relative difference in mobilization of monocytes between hypoglycemia and
275 controls. Eight subjects were recruited per group to allow for a 13% drop out rate. Mean
276 baseline measurements of glucose were compared between groups using analysis of variance
277 (ANOVA). Mean measurements of glucose, insulin, and epinephrine at 60 minutes post
278 clamp were compared, adjusting for clamp baseline measurement, using analysis of
279 covariance (ANCOVA). In the event of unequal variance between groups, a log transform
280 was applied and the analysis repeated. Longitudinal and between-group comparisons were
281 made for post-endotoxin measurements using mixed effects linear models. For models
282 examining between-group differences, the baseline endotoxin measurement was included as a
283 covariate. For all mixed-effects linear models, an autoregressive correlation structure (AR1)
284 was used to allow for the correlation between multiple measurements on the same person.
285 Planned contrasts were made versus baseline and between groups at equivalent time points
286 with Sidak's correction for multiple comparisons. All data are expressed as mean ± SEM,
287 unless otherwise specified, and a P value of < 0.05 was deemed statistically significant.
288 Analysis was performed using SPSS (version 22.0, IBM, Chicago, Illinois, USA).

289 Results

290 **Participants**

291 Study participants across the three groups were well matched for age, sex, BMI, HbA_{1C} and
292 total WBC count with no significant differences at screening (Table 1). Participant numbers
293 at each stage of study is illustrated in a flow diagram (Supplemental flow diagram).

294

295 Clamp studies

296 Glucose, insulin and counter regulatory hormones

297 Arterialized blood glucose values are shown in Fig. 1a. The glucose values were 2.51 ± 0.11
298 mmol/l and 6.04 ± 0.16 mmol/l at the end of the hypoglycemia and euglycemia clamps,
299 respectively. Glucose values at the end of the sham-saline clamp were 4.64 ± 0.09 mmol/l. A
300 counterregulatory response to hypoglycemia was evident with epinephrine levels during
301 hypoglycemia (1.87 ± 0.25 nmol/l) being significantly higher ($P < 0.001$) compared to
302 euglycemia (0.07 ± 0.01 nmol/l) and sham-saline (0.10 ± 0.04 nmol/l) (Fig. 1b). Free insulin
303 levels at the end of clamp were similar between hypoglycemia (968.5 ± 149.1 pmol/l) and
304 euglycemia groups (1025.4 ± 81.4 pmol/l, $P = 0.996$) but significantly higher ($P < 0.001$)
305 then those in the sham-saline (31.3 ± 6.3 pmol/l) group (Fig. 1c). Serum cortisol and growth
306 hormone were significantly higher in the hypoglycemia group compared to euglycemia and
307 sham-saline controls (Fig. 1d and 1e).

308

309 Total and differential leucocyte count

310 We determined if hypoglycemia results in changes in circulating leucocytes. Hypoglycemia
311 significantly increased the total number of WBC compared to controls (Fig. 2a). There was
312 an increase across all classes of leucocytes studied, including neutrophils (Fig. 2b),
313 lymphocytes (Fig. 2c) and total monocytes (Fig. 3a).

314

315 Monocyte subsets

316 We sought to determine if hypoglycemia exerted specific effects on monocyte subsets
317 associated with cardiac pathology. Hypoglycemia increased the absolute number of all three
318 circulating monocyte subsets compared to euglycemia and sham-saline (Fig. 3b-d). The

319 number of circulating NCM after 60 minutes of hypoglycemia compared to baseline (17.6 ±
320 2.9 cells/μl) increased twofold. IM numbers after 60 minutes of hypoglycemia compared to
321 baseline (23.2 ± 4.5 cells/μl) increased by a factor of 1.81 and CM after 60 minutes of
322 hypoglycemia compared to baseline (442.4 ± 55.3 cells/μl) increased by a factor of 1.29.
323 There were no significant differences in the baseline values of all three monocyte subsets
324 between the study groups.

325

326 **Platelet count, aggregation and monocyte-platelet aggregates**

327 Activation of platelets and generation of platelet-leucocyte aggregates contribute to leucocyte
328 mobilization and inflammation in the vasculature (36). We therefore studied platelet number
329 and function and their interaction with leucocytes. Total platelet count increased in
330 hypoglycemia compared to euglycemia and sham-saline controls (Fig. 4a). ADP-induced
331 platelet aggregation increased following 60 minutes of hypoglycemia versus euglycemia (P =
332 0.014) and there was numerically, but not statistically significantly, higher platelet
333 aggregation detected in the hypoglycemia group compared to sham-saline group ($P = 0.064$)
334 (Fig. 4b). The total number of MPA increased following 60 minutes of hypoglycemia
335 compared to euglycemia (Fig. 4c). Whilst total MPA were not significantly higher in
336 hypoglycemia compared to sham-saline controls at 60 minutes (Fig. 4c), we observed specific
337 increases in non-classical monocyte (NCM) and intermediate monocyte (IM)-platelet
338 aggregates (Fig. 4d and 4e). Classical monocyte (CM)-platelet aggregates appeared to
339 increase following 60 minutes of hypoglycemia versus euglycemia and sham-saline but this
340 was not statistically significant ($P = 0.054$) (Fig. 4f).

341

342

343

344

345 **Cell surface markers**

346 To further explore the activation state of monocytes after hypoglycemia, we studied
347 expression levels of chemokine receptor CX_3CR1 and integrin CD11b. Hypoglycemia did not
348 alter the expression of CX_3CR1 or CD11b (Supplemental Fig. 1a and 1b).

349

350 ## Endotoxin challenge

351 To determine if prior hypoglycemia affected the subsequent response to a classical immune
352 activator, and thus to reveal if hypoglycemia had any longer-lasting effects on the innate
353 immune system, we next proceeded to a low-dose intravenous endotoxin challenge 48 hours
354 after the hypoglycemic challenge in all subjects. Consistent with the low-dose model
355 employed, no fever or significant change in mean arterial blood pressure was recorded
356 following endotoxin challenge across the study groups.

357

358 **Epinephrine, cortisol and growth hormone**

359 In contrast to the stress response induced by hypoglycemia, epinephrine levels were not
360 significantly different between study groups 6 hours following endotoxin administration (Fig.
361 5a). In the hypoglycemia group, epinephrine levels were 0.15 ± 0.04 nmol/l versus $0.06 \pm$
362 0.01 nmol/l in euglycemia group and 0.09 ± 0.01 nmol/l in sham-saline group. There were
363 also no differences detected between groups in serum cortisol and growth hormone levels
364 following endotoxin administration (Fig. 5b and 5c). However, a rise versus baseline in the
365 stress hormone cortisol was evident whereby serum cortisol levels peaked at 4 hours
366 following endotoxin challenge in all study groups ($P = 0.005$) (Fig. 5b).

367

368

369

370 **Total and differential leucocyte count**

371 We observed that antecedent hypoglycemia modulated the subsequent WBC response to
372 endotoxin. Total number of WBC increased significantly following endotoxin in all the study
373 groups (Fig. 6a). The peak WBC response occurred at 4 hours post endotoxin and this was
374 significantly higher in the hypoglycemia group at 10.96 ± $0.97x10^9$/l vs 8.21 ± $0.85x10^9$/l in
375 the euglycemia group (P = 0.012) (Fig. 6a). Total WBC count 4 hours post endotoxin in the
376 sham-saline group was 10.65 ± $0.64x10^9$/l and this was significantly higher compared to
377 euglycemia (P = 0.033), but not hypoglycemia (P = 0.974). The rise in WBC was mainly a
378 consequence of an increase in neutrophil count (Fig. 6b). The lymphocyte count decreased
379 following endotoxin (Fig. 6c) and the monocyte count initially decreased prior to recovery 6
380 hours post endotoxin (Fig. 7a). There was a trend towards a higher total monocyte count in
381 the hypoglycemia group 4 hours post endotoxin compared to euglycemia but this comparison
382 did not reach statistical significance (P = 0.085). The absolute number of circulating
383 monocyte subsets did not differ significantly between study groups (Fig. 7b, 7c and 7d).
384 NCM and IM numbers decreased significantly following endotoxin compared to baseline
385 values in all groups (P < 0.001) (Fig. 7b and 7c). Compared to baseline, CM numbers
386 significantly declined at 2 hours (P < 0.001), prior to rising and reaching a peak at 6 hours (P
387 < 0.001) (Fig. 7d).

388

389 **Cell surface markers**

390 We examined monocyte activation following endotoxin exposure by measurement of cell
391 surface marker CX_3CR1 expression. Endotoxin administration caused a significant decline in
392 expression of this marker across all study groups compared to baseline (P < 0.001)
393 (Supplemental Fig. 2a). This was accompanied by an increase in the concentration of

394 CX_3CL1 in plasma at 4 and 6 hours compared to baseline in all groups ($P < 0.001$)
395 (Supplemental Fig. 2e). Activation of monocytes was also revealed by increased expression
396 of CD11b expression at 4 and 6 hours following endotoxin compared to baseline in all groups
397 ($P < 0.001$) (Supplemental Fig. 3a). In addition, the percentage of total monocytes that were
398 positive for CD11b expression was higher in hypoglycemia group versus euglycemia group at
399 2 hours post endotoxin ($P = 0.007$) (Supplemental Fig. 3b).

400 # Discussion

401 Hypoglycemia may contribute to exacerbations of ischemic CV disease. We aimed to
402 investigate the effect of acute experimental hypoglycemia and subsequent low-dose
403 endotoxemia on aspects of the innate immune response (total leucocytes, leucocyte subsets
404 and specifically monocyte subsets), thrombosis (platelet aggregation) and cross-talk between
405 inflammation and thrombosis (monocyte-platelet aggregates). Our main findings were: (1)
406 hypoglycemia increased the number of all three circulating monocyte subsets, in association
407 with a stress response characterized by increased plasma epinephrine levels; (2)
408 hypoglycemia increased platelet reactivity, promoted formation of MPAs and promoted
409 aggregate formation between proinflammatory monocytes and platelets; (3) leucocyte
410 mobilization to the stress response of low-dose endotoxin was independent of epinephrine,
411 and antecedent hypoglycemia resulted in a significantly higher inflammatory leucocyte
412 response to low-dose endotoxin administered 48 hours later.
413
414 As shown previously (13, 37), we confirm that hypoglycemia results in leucocytosis. In
415 addition, we present, for the first time, the effect of hypoglycemia on monocyte subset
416 kinetics and demonstrate an increase in the absolute number of all three circulating monocyte
417 subsets. The largest increase was observed in numbers of circulating NCM (2-fold) and IM

418 (1.8-fold) with a modest increase in the number of CM (1.3-fold). These data are in keeping
419 with an observed selective mobilization of CD16$^+$ monocytes in response to exercise (27, 38)
420 and epinephrine infusion (39). Ratter et al. also recently determined that hypoglycemia might
421 modify selective monocyte mobilization (37). However, they did not phenotype monocyte
422 subsets but rather measured total levels of CD16 on peripheral blood mononuclear cells
423 isolated from both healthy participants and those with type 1 diabetes in experimental
424 hypoglycemia settings. Our data identify specific changes in monocyte subsets that have been
425 previously linked to monocyte activation and atherogenesis. As observational data support
426 the notion of CD16$^+$ monocytes being proatherogenic (20, 22-24), and adrenergic modulation
427 of monocytes induces proinflammatory changes (40), an increase in the circulating number of
428 these cells following hypoglycemia may enhance CV risk in diabetes.
429
430 Previous studies investigating effects of hypoglycemia on platelet biology have suggested an
431 increase in platelet reactivity; however, this was in the context of significant hypoglycemic
432 stimulus as part of an insulin stress test (41). An older investigation into the effect of
433 hypoglycemia on monocyte-platelet interactions in type 1 diabetes and healthy controls has
434 also suggested a trend towards increased MPA formation, but these data were not conclusive
435 with little difference between euglycemic and hypoglycemic conditions (42). Our study also
436 recapitulates and extends previous findings that hypoglycemia is prothrombotic, as evidenced
437 by an increased platelet count and increased platelet reactivity to ADP (43). We have now
438 conclusively demonstrated an overall increase in formation of MPA in hypoglycemia in
439 comparison to euglycemia. Furthermore, we provide novel data demonstrating MPA
440 formation within monocyte subsets in experimental hypoglycemia. MPA formation is a
441 highly sensitive marker of both monocyte and platelet activation (44, 45). MPA formation
442 promotes monocyte release of the proinflammatory cytokines; TNFα, CXCL8 and CCL2 (46,

443 47) and increases adhesive properties of monocytes (48), thereby representing a bridge
444 between inflammation and thrombosis, that may serve to increase CV risk. In acute coronary
445 syndromes, MPA formation correlates with troponin elevation, risk of in-hospital cardiac
446 events including death and risk of future cardiac events (20, 49). We have also shown that
447 NCM and IM aggregate more readily with platelets in response to hypoglycemia compared to
448 CM. A similar observation of proportionally higher IM-MPA and NCM-MPA formation has
449 been reported in patients following an ST-elevation myocardial infarction (STEMI) with
450 higher IM-MPAs in particular being a poor prognostic indicator at 6 weeks following STEMI
451 (20). Thus, our data suggest that hypoglycemia not only increased circulating numbers of
452 $CD16^+$ monocytes, but also promoted increased interaction between these proinflammatory
453 monocyte subsets and platelets.
454
455 In a first model of its type, we wished to determine whether antecedent hypoglycemia
456 modulated responses to low-dose endotoxin. We chose a low-dose endotoxin model firstly
457 because we felt it the safest way to combine the clamp and endotoxin human models,
458 secondly because future extension to the study of people with diabetes would be more
459 feasible with this model, and finally because people with diabetes are often exposed to
460 chronic low-grade infections through foot ulceration and periodontitis which might further
461 increase the risk of CV mortality (50, 51). In our model, we observed in all groups that
462 monocytes were activated even in response to low-dose endotoxin, as indicated by
463 upregulation of systemic levels of the CX_3CR1 ligand CX_3CL1, and the upregulation of the
464 adhesion molecule CD11b on the monocytes themselves. Interestingly, compared to
465 euglycemia, hypoglycemia resulted in greater leucocyte mobilization in response to low-dose
466 intravenous endotoxemia 48 hours later. Furthermore, we noted a non-significant trend
467 towards a higher total monocyte count in the hypoglycemia group 4 hours post endotoxin

468 compared to euglycemia. The percentage of monocytes that were CD11b positive was also
469 higher in hypoglycemia group compared to euglycemia group at 2 hours post endotoxin.
470 Levels of leucocyte mobilization were similar between groups who received prior sham-
471 saline or hypoglycemia. These data suggest that euglycemia with insulin suppressed
472 leucocyte mobilization in response to endotoxin 48 hours later, consistent with the known
473 antiinflammatory actions of insulin (52, 53) and that the physiological stress of hypoglycemia
474 overcame this insulin-mediated suppression of inflammatory responses. Our data show that
475 drivers for differential leucocyte mobilization to endotoxin are unlikely to be due to
476 differences between groups in epinephrine, cortisol and growth hormone levels post
477 endotoxin as these were not significantly different. Our observation that a single episode of
478 hypoglycemia compared to euglycemia invokes a stronger proinflammatory response to
479 endotoxin up to 2 days later is of potential clinical relevance given that trial data suggest
480 downstream mortality following hypoglycemia (29-31).
481
482 The strengths of our study include use of a novel human experimental model and detailed
483 flow cytometric analysis that allowed us to comprehensively describe immune cell kinetics
484 and activation status in response to experimental hypoglycemia and endotoxin challenge *in*
485 *vivo*. The separation of clamp and endotoxin studies by 48 hours allowed us to probe the
486 longitudinal effects of hypoglycemia on innate immunity. Moreover, by using a sham-saline
487 group, we specifically controlled for the immunological effects of insulin, thereby robustly
488 investigating proinflammatory changes in response to hypoglycemia.
489
490 One limitation was our decision to study a relatively small number of young healthy
491 participants. This limits the applicability of our findings to older patients with diabetes,
492 established CV risk factors and atherosclerosis. For ethical and safety reasons, we decided to

493 examine our novel experimental model initially in healthy participants. We also specifically
494 adopted a low-dose endotoxin model with future translatability in older, higher-risk
495 participants in mind. Future studies should therefore confirm our findings in those with
496 diabetes. In addition, it is worth noting that we studied cell numbers, phenotypic changes and
497 activation status in circulating immune cells and this may not necessarily reflect the
498 functional capacity of these cells in an atherosclerotic plaque. An animal model of combined
499 experimental hypoglycemia and atherosclerosis may help to resolve these questions.
500

501 In conclusion, hypoglycemia mobilized proatherogenic monocyte subsets and induced
502 prothrombotic changes by increasing platelet reactivity. In addition, hypoglycemia amplified
503 interactions between platelets and monocytes by promoting MPA formation with enhanced
504 aggregation of proinflammatory monocytes with platelets. Hypoglycemia may also prime the
505 innate immune system to respond more robustly to stimuli such as endotoxin. This implies
506 proinflammatory consequences of hypoglycemia beyond the acute episode. These data
507 provide novel mechanistic insights into how hypoglycemia could increase CV risk through
508 upregulation of inflammatory responses.

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517

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519

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Table & Figure legends

Table 1: Comparison of participant characteristics at baseline. Data are mean ± SD or median (interquartile range). *P* values indicate comparisons between study groups via parametric or nonparametric testing. BMI, body mass index; WBC, white blood cells.

Figure 1: Glucose, insulin and counter-regulatory hormones in clamp studies. Arterialized whole blood glucose values during hyperinsulinemic hypoglycemic, euglycemic and sham-saline clamps (a), epinephrine (b), free insulin (c), cortisol (d) and growth hormone values (e) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), *P<0.05, **P<0.01, ***P<0.001, ns-non-significant, P-values are provided for comparison between study groups. Black circles (and dashed line 1a)-hypoglycemia group; open circles-euglycemia group; black triangles (and solid line 1a)-sham-saline group.

Figure 2: Peripheral total white blood cell, neutrophil and lymphocyte kinetics in experimental hypoglycemia and controls. Number of circulating total WBCs (a), neutrophils (b) and lymphocytes (c) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), **P<0.01, ***P<0.001, P-values are provided for comparison between study groups. Black circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group. WBC, white blood cells.

Figure 3: Total monocyte count and monocyte subset kinetics in experimental hypoglycemia and controls. Absolute circulating numbers of total monocytes (a) and monocyte subsets comprising of NCM (b), IM (c) and CM (d) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), *P<0.05, **P<0.01, ***P<0.001, P-values are provided for comparison between study groups. Black

circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical monocytes.

Figure 4: Platelet reactivity and monocyte-platelet aggregate formation in experimental hypoglycemia and controls. Total platelet count (a), platelet aggregation to ADP 6.45 μM (b), total MPA formation (c) and MPA formation within monocyte subsets; NCM-MPA (d), IM-MPA (e) and CM-MPA (f) after 60 minutes of hypoglycemia, euglycemia or sham-saline injection. Data are mean (SEM), *P<0.05, **P<0.01, ns-non-significant, P-values are provided for comparison between study groups. Black circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group. MPA, monocyte-platelet aggregates; NCM-MPA, non-classical monocyte-platelet aggregates; IM-MPA, intermediate monocyte-platelet aggregates; CM-MPA, classical monocyte-platelet aggregates.

Figure 5: Changes in epinephrine, cortisol and growth hormone response post endotoxin challenge. Epinephrine (a), cortisol (b) and growth hormone (c) responses 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin challenge in participants that underwent hypoglycemia, euglycemia or a sham-saline clamp 48 hours earlier. Data are mean (SEM), **P<0.01, ns-non-significant, P-value on dashed line in (b) represents change in cortisol at 4 hours compared to baseline in all groups, solid horizontal lines represent significance for comparison between study groups. Dashed line in (a) illustrates the mean epinephrine response in hypoglycemia clamp subjects. Black circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline group.

744 **Figure 6: Peripheral total white blood cell, neutrophil and lymphocyte kinetics post**
745 **endotoxin challenge.** Number of circulating total WBCs (a), neutrophils (b) and
746 lymphocytes (c) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous endotoxin
747 challenge in participants that underwent hypoglycemia, euglycemia or a sham-saline clamp
748 48 hours earlier. Data are mean (SEM), *P<0.05, ns-non-significant, P-values are provided
749 for comparison between study groups, solid horizontal line in (c) represents significance for
750 comparison between study groups. Black circles-hypoglycemia group; open circles-
751 euglycemia group; black triangles-sham-saline group.
752
753 **Figure 7: Total monocyte count and monocyte subset kinetics post endotoxin challenge.**
754 Absolute circulating numbers of total monocytes (a) and monocyte subsets comprising of
755 NCM (b), IM (c) and CM (d) 2, 4 and 6 hours following low dose (0.3 ng/kg) intravenous
756 endotoxin challenge in participants that underwent hypoglycemia, euglycemia or a sham-
757 saline clamp 48 hours earlier. Data are mean (SEM), ***P<0.001, ns-non-significant, P-value
758 on dashed line in (b) represents change in number of NCMs at 2, 4 and 6 hours compared to
759 baseline in all study groups. P-value on dashed line in (c) represents change in number of IM
760 at 2, 4 and 6 hours compared to baseline in all study groups. P-value on dashed lines in (d)
761 represent change in number of CM at 2 and 6 hours compared to baseline in all study groups.
762 Solid horizontal lines represent significance for comparison between study groups. Black
763 circles-hypoglycemia group; open circles-euglycemia group; black triangles-sham-saline
764 group. NCM, non-classical monocytes; IM, intermediate monocytes; CM, classical
765 monocytes.
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Parameter	Hypoglycemia	Euglycemia	Sham-saline	*p*
Total *n=24*	8	8	8	N/A
Sex (M/F)	4/4	4/4	4/4	N/A
Age (years)	21 (19-22)	21 (20-23)	21.5 (21-26)	0.299
BMI (kg/m^2)	24 ± 2	23 ± 2	24 ± 4	0.638
HbA_{1c}				
%	5.2 ± 0.31	5.2 ± 0.26	5.1 ± 0.14	0.792
mmol/mol	34 ± 3.6	33.5 ± 2.8	32.6 ± 1.4	0.616
Total WBC ($x10^9$/l)	6.26 ± 1.42	4.83 ± 0.91	4.50 ± 1.69	0.102

Figure
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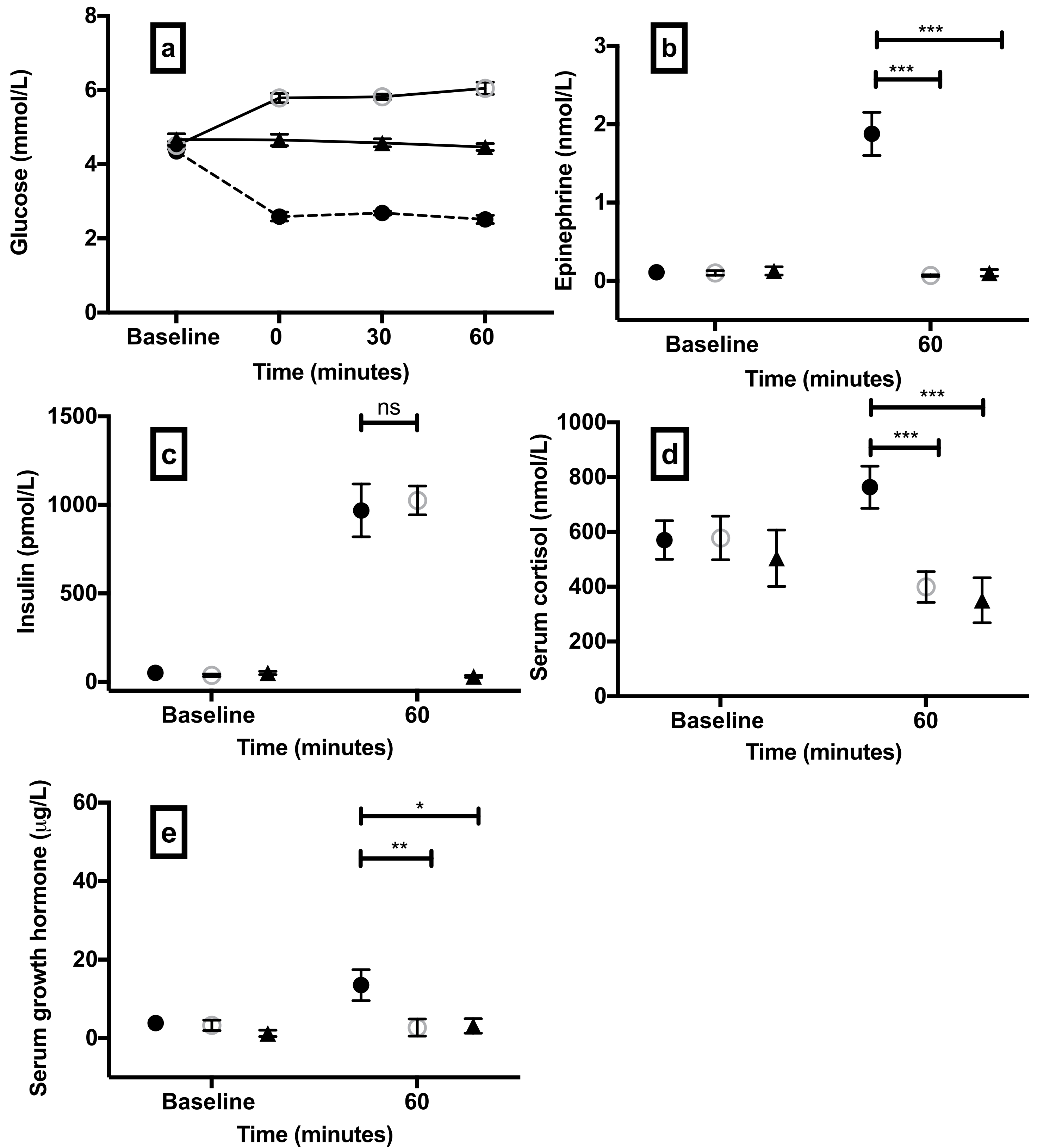

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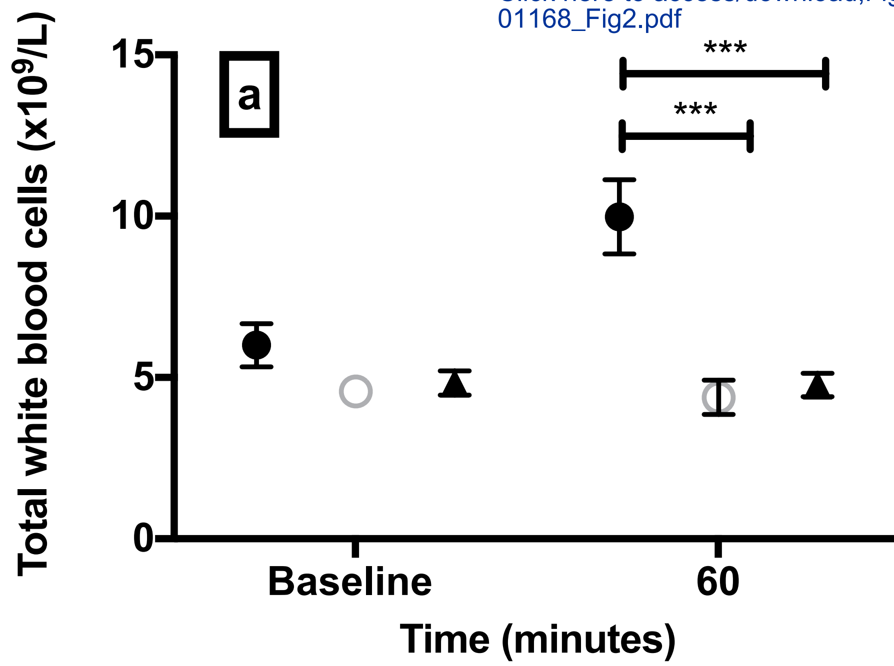

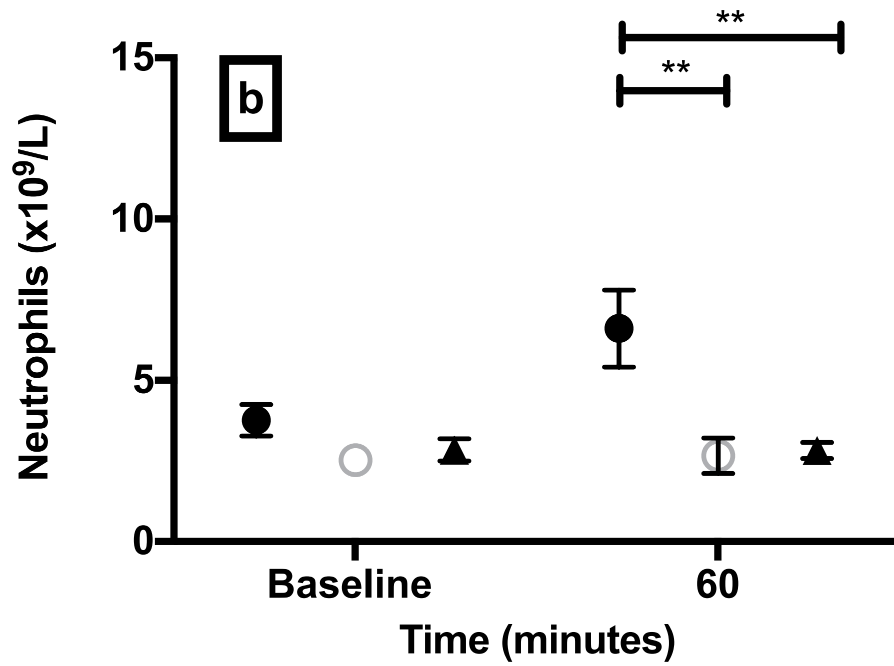

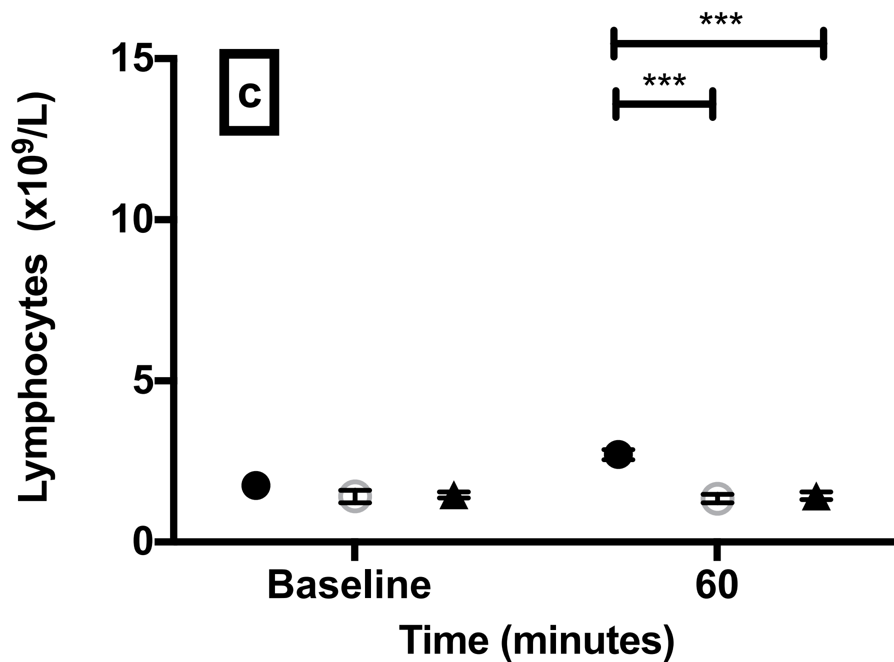

Figure

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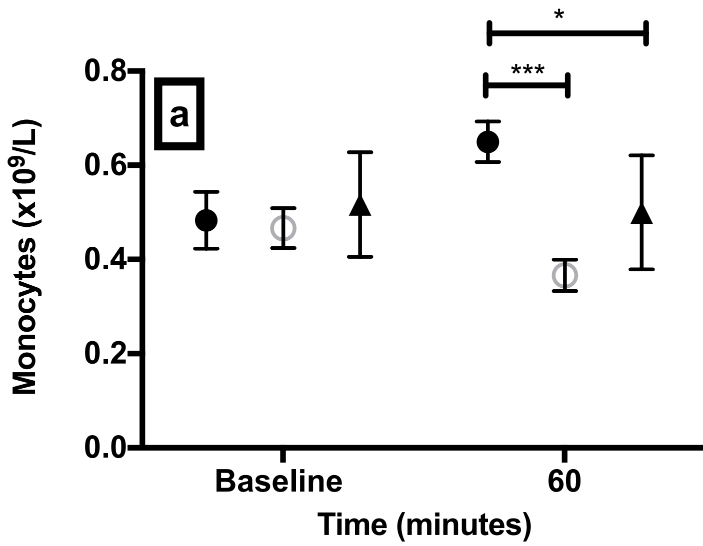

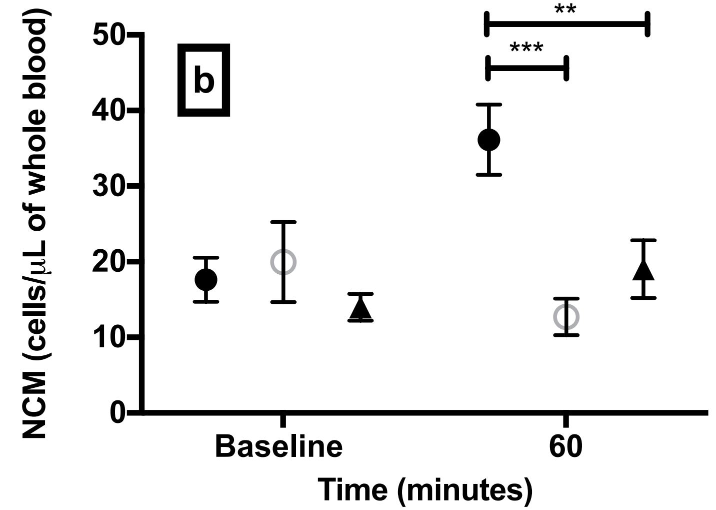

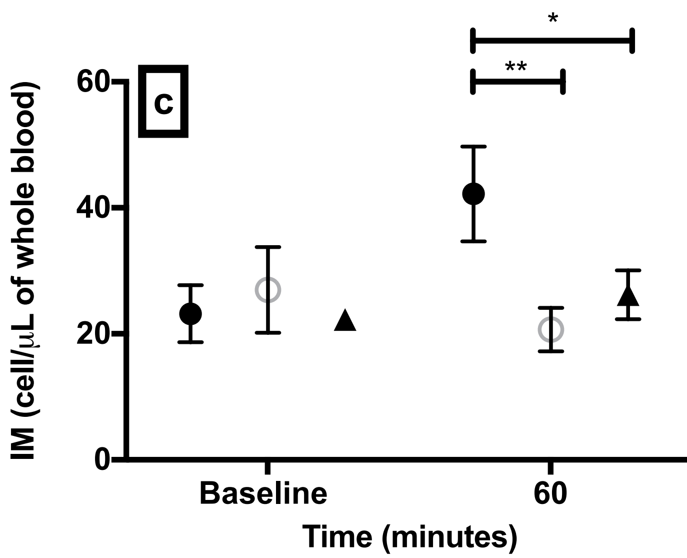

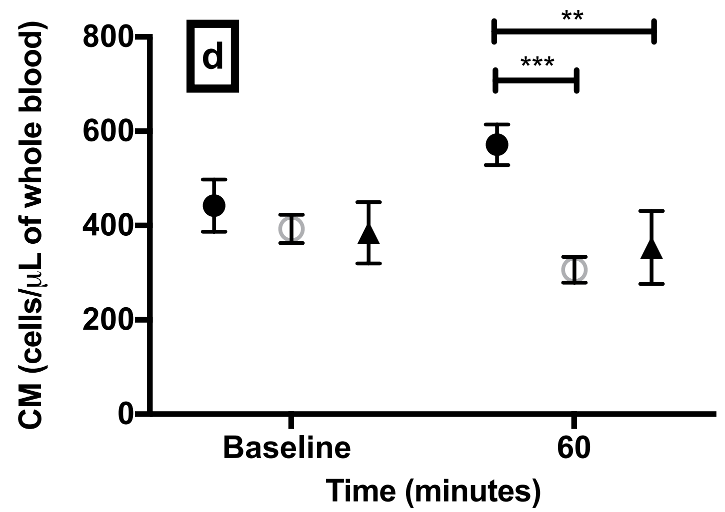

Figure

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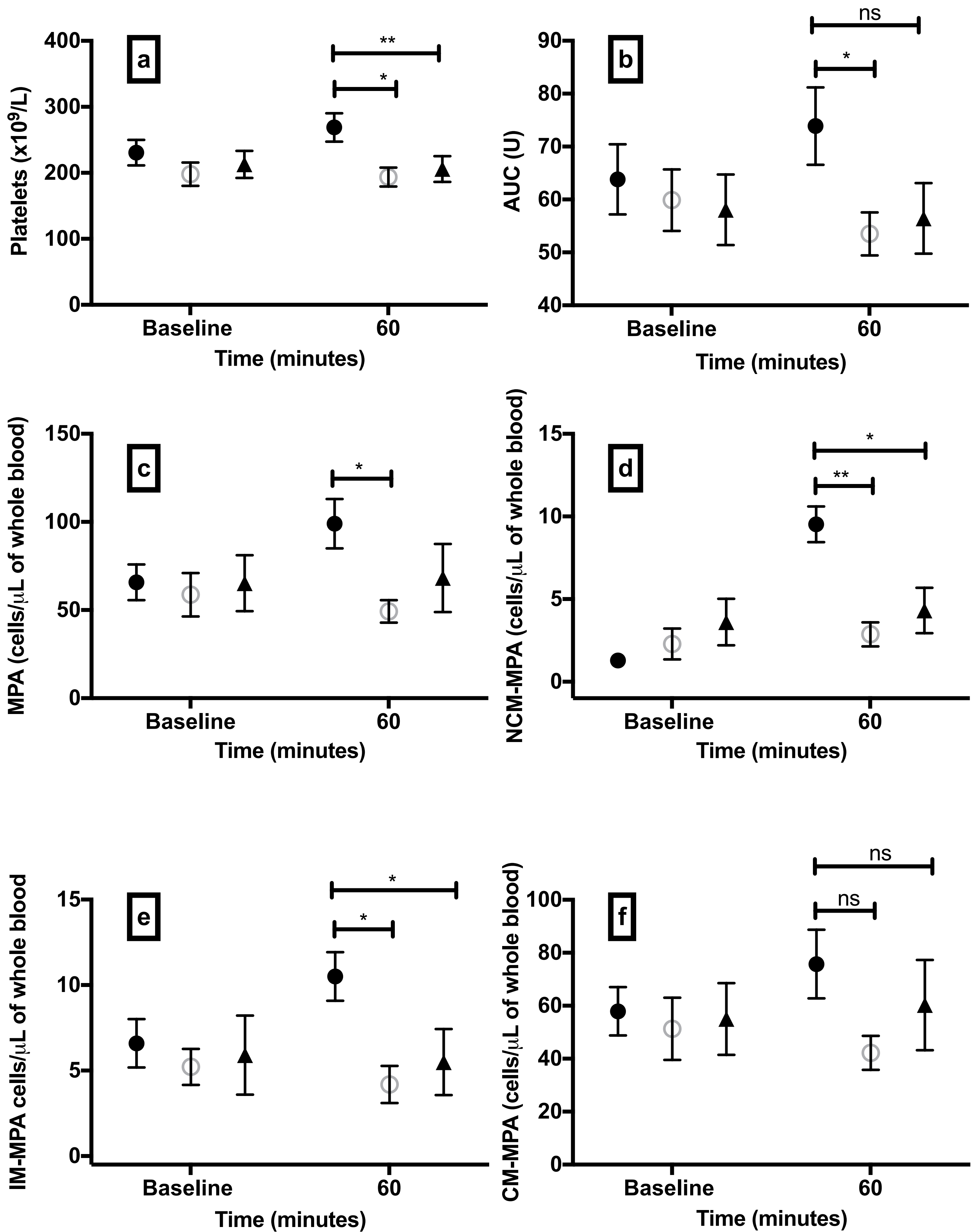

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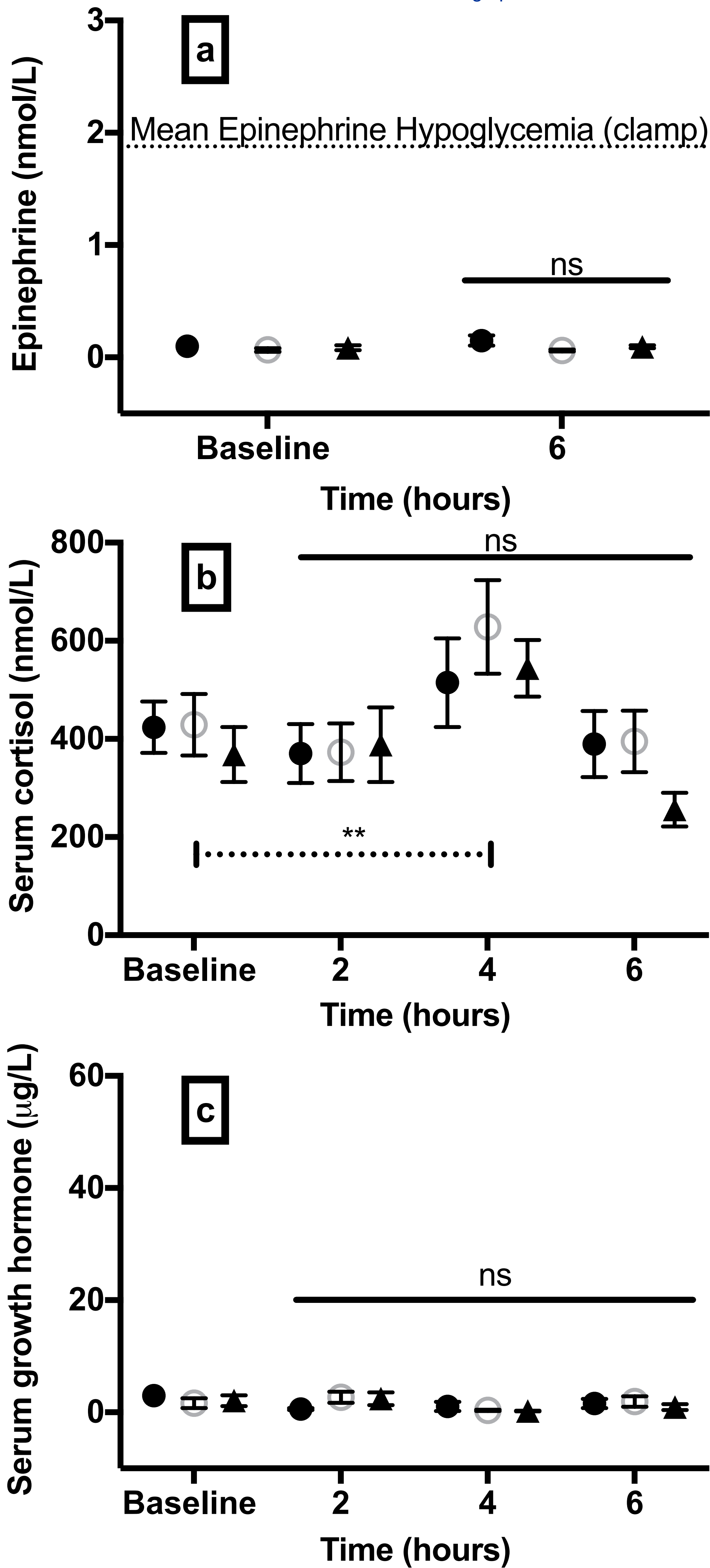

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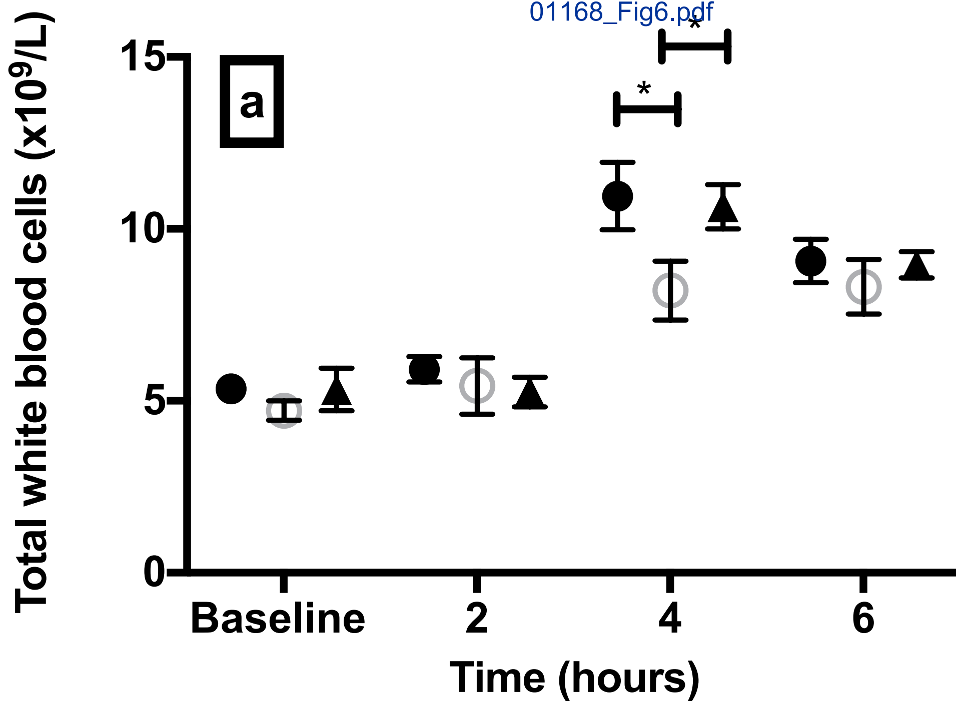

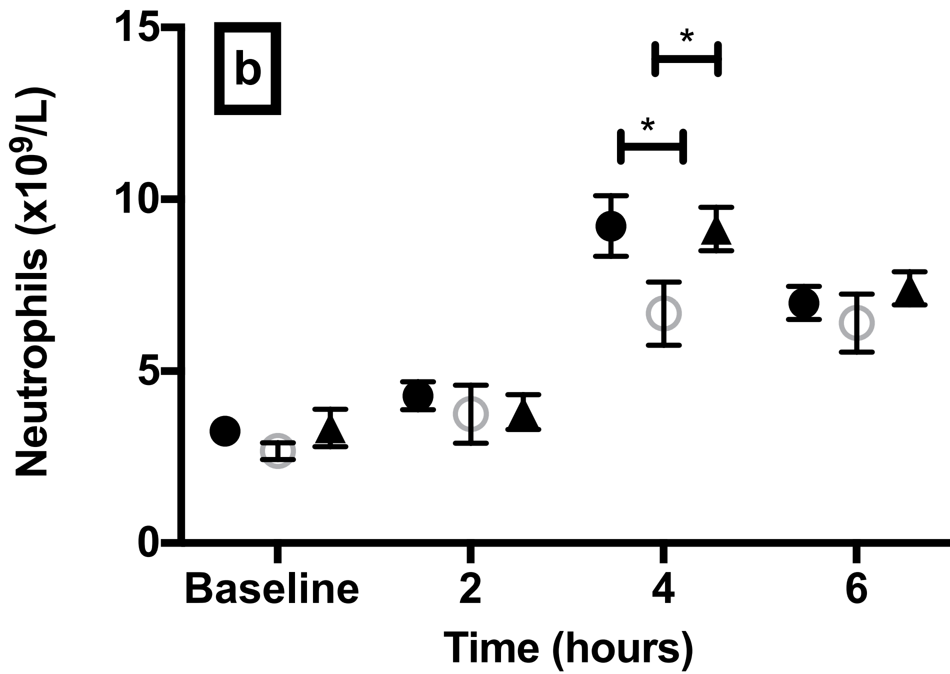

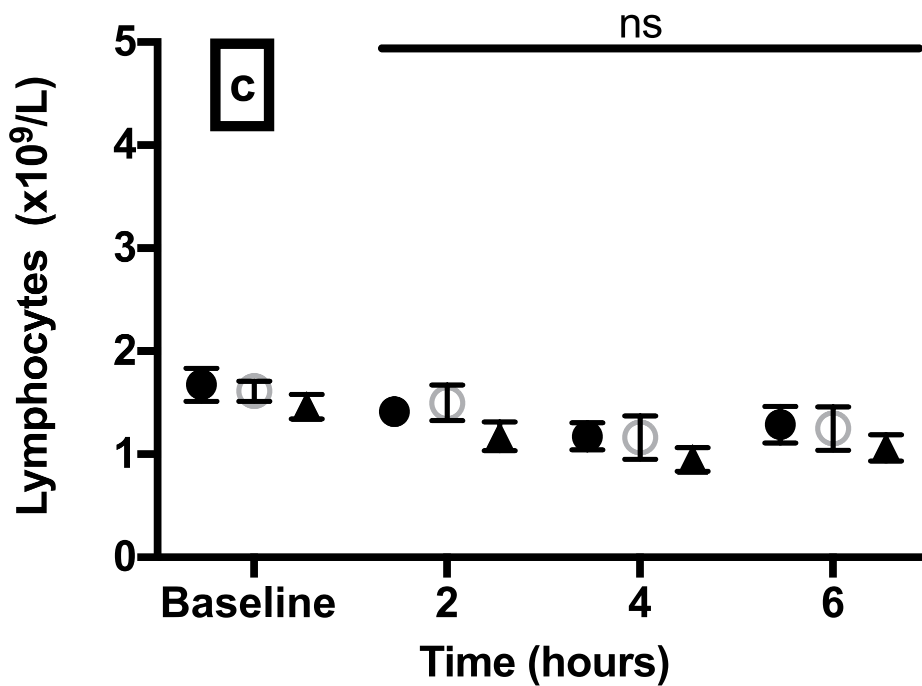

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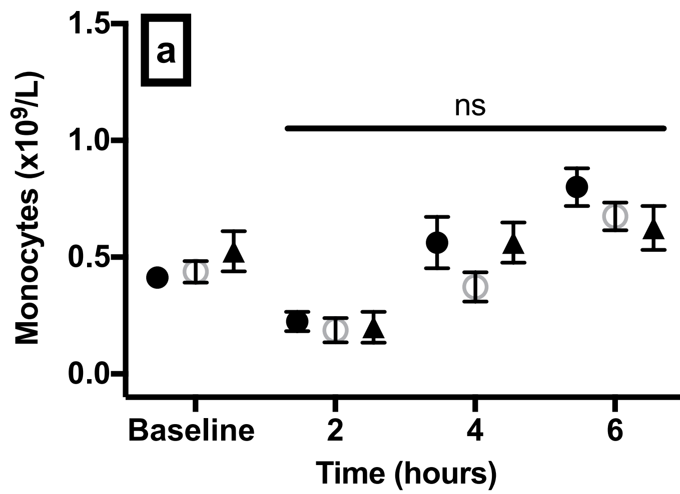

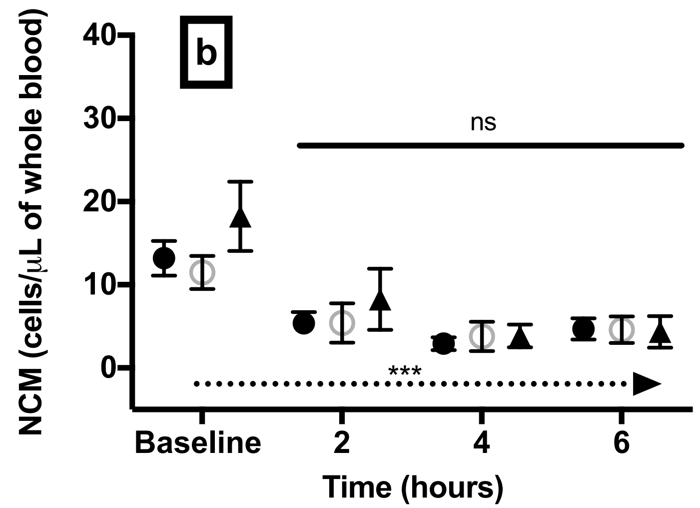

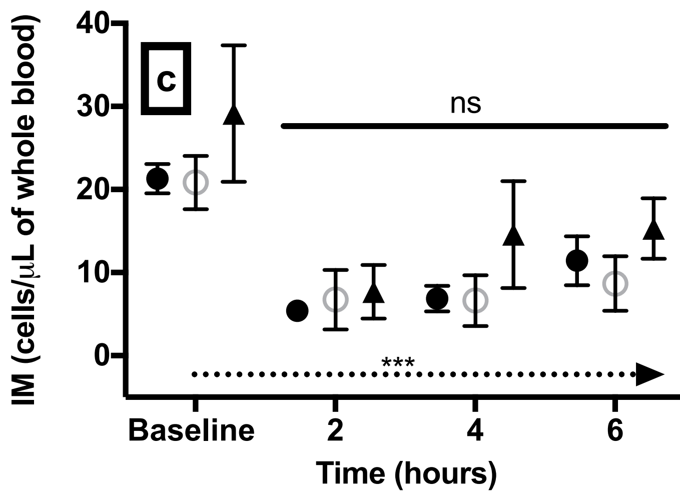

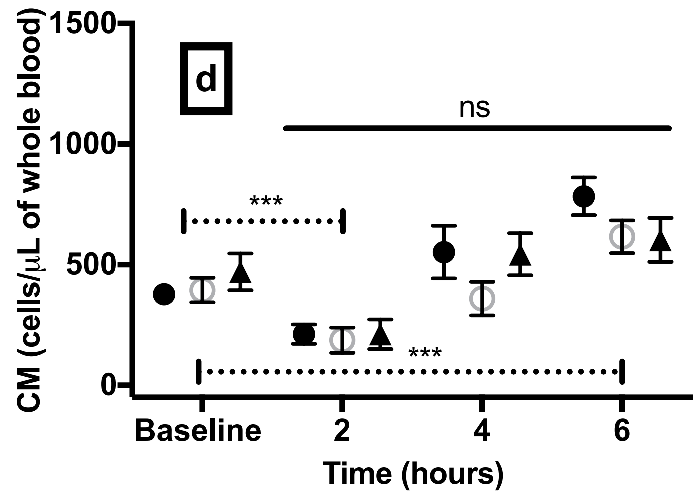

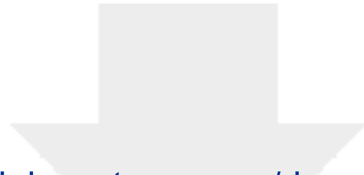

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