Maternal High-fat Diet During Pregnancy Programs Platelet Hyperactivation in Male Mouse Offspring

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Abstract

Background: Maternal over-nutrition increases the risk of diabetes and cardiovascular events in offspring. While prominent effects on cardiovascular health are observed, the impact of this on platelet physiology has not been studied. Here, we sought to determine whether maternal high-fat diet (HF) ingestion can affect the platelet function in offspring.

Methods: C57BL6/N mice dams were given a HF or control (C) diet for 8 weeks prior to and during pregnancy. Male offspring also received either C or HF diets for 26 weeks. Experimental groups were: C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C and HF/HF. Various phenotypic (including body weight and % of body fat) and metabolic (glycaemia, triglyceridemia) tests were performed and blood collected for platelet studies.

Results: Compared to C/C, HF/HF animals were obese, with fat accumulation, hyperglycaemia, insulin resistance and low respiratory exchange rate. HF/HF, but not C/HF mice also showed hypertriglyceridaemia and higher mean platelet volume. These platelets were hyperreactive, displaying higher fibrinogen binding after stimulation with different agonists. They also showed increased platelet adhesion and spreading on collagen. Maternal obesity led to an overall effect of increased platelet reactivity in offspring. Both maternal and offspring HF groups presented decreased levels of collagen receptor GPVI with increased oxidative stress. Western blotting experiments in stimulated platelets showed increased phosphorylation of PKC substrates, total tyrosine and AKT at Ser473, whilst response to nitric oxide donor PAPA-NONOate was unchanged compared to C/C.

Conclusions: Maternal HF diet ingestion programmes platelet hyperactivation in male mouse offspring, whilst HF in both dams and offspring resulted in a ‘double-hit’ effect of increased serum triglyceride levels, large platelets and increased reactivity. This involved enhanced Tyr phosphorylation, ROS production and decreased GPVI expression. Since platelet function can be programmed by early developmental periods, it is possible to use this window of intervention to reduce the risk of thrombotic events.

1. Background

The concept that the in utero environment impacts long-term metabolic health was first introduced in the late 1980s by Prof David Barker at the University of Southampton [1]. Analysing data from large cohort studies, he noted that infants of low birth weight (a proxy for in utero malnutrition), had increased risk of developing cardiovascular disease and dying of acute thrombotic events (such as stroke and myocardial infarction) in adulthood [2, 3]. This concept of developmental programming of cardiovascular disease has since been expanded to include maternal over-nutrition, which is more reflective of Western lifestyles. Both epidemiological and animal studies have established unequivocally that maternal diabetes, obesity or maternal high-fat (HF) diet during pregnancy is associated with increased susceptibility to
cardiovascular disease in offspring (reviewed by Williams et al [4]), although the precise mechanisms underlying this observation remain to be established.

A key component of cardiovascular disease pathophysiology is platelet reactivity, which has been somewhat neglected in the developmental programming field. Platelets contribute to the initiation and development of atherosclerosis through their interaction with activated endothelium and inflammatory cells [5–7]. From a clinical perspective however, their major role is at the later stages of disease when platelets can form unwanted thrombi on atherosclerotic plaques, or when unstable plaques rupture. These acute thrombotic events cause stenosis or occlusion of vessels supplying the heart or brain resulting in myocardial infarction (MI) or stroke respectively (for review see [8]). To this end, a recent cohort study has documented that increased platelet activation in participants before the onset of cardiovascular disease, was a major risk factor and predictor for myocardial infarction and stroke events later in life [9].

Various components of the cardiovascular system of offspring appear to be negatively affected by in utero over-nutrition. Studies in sheep and rodents documented that maternal diet-induced obesity during pregnancy led to endothelial dysfunction, cardiac remodelling, cardiac inflammation and fibrosis, cardiac hypertrophy and impaired cardiac contractility in offspring [10–13]. No measurement of platelet function was reported in those studies. The concept that cardiovascular disease in later life might be programmed during development by maternal over-nutrition is particularly alarming given that the UK has the highest rate of obesity amongst women of reproductive age in Europe [14]. Likewise, maternal diabetes during pregnancy increased early onset of cardiovascular disease in offspring [15].

Despite strong evidence for developmental programming of cardiovascular disease and the key role of platelets on cardiovascular disease pathophysiology, there is currently no literature assessing the effects of maternal metabolic dysfunction on the platelet reactivity of the offspring. Using a mouse model, we set out to examine the effects of maternal HF diet ingestion on the platelet function of 30-week-old male offspring mice. Our data showed an overall effect of increased platelet reactivity on offspring born to HF dams and a worsened phenotype if offspring were also fed HF. This suggests 1) that platelet function is programmed by maternal obesity and 2) that there is a ‘double-hit’ effect in which metabolic dysfunction in both mother and offspring potentiate platelet reactivity. This is the first study to describe the deleterious impact of maternal HF ingestion on the platelet reactivity of the offspring and offer a possible pathophysiological mechanism to previous reports showing increased cardiovascular events related to maternal metabolic dysfunction.

2. Methods

2.1. Reagents

Prostacyclin (PGI₂), Adenosine Diphosphate (ADP), thrombin and 2’7’-Dichlorofluorescin diacetate (DCFDA) were purchased from Sigma-Aldrich (Dorset, UK). PAPA-NONOate was purchased from Tocris...
FITC-conjugated fibrinogen was purchased from Agilent (Stockport, UK). Collagen was purchased from Nycomed (Munich, Germany) and Collagen-Related Peptide (CRP) was obtained from Prof Richard Farndale (University of Cambridge, Cambridge, UK). Alexa-488 conjugated phalloidin was purchased from Life Technologies (Paisley, UK). Rat anti-mouse GPVI, α2 integrin, GpIbα and appropriate IgG controls were purchased from Emfret (Emfret Analytics GmbH & Co, Eibelstadt, Germany). Goat anti-mouse CD36 was purchased from R&D Systems (R&D Systems Inc, Abingdon, UK). Anti-phospho Tyr 4G10 antibody was purchased from MercK Millipore (Watford, UK). Polyclonal goat anti-β-Actin antibody was purchased from Abcam (Cambridge, UK). Anti-phospho-vasodilator-stimulated phospho-protein (VASP) Ser239 and anti-protein kinase B (Akt) Ser473 were purchased from Cell Signalling (Hitchin, UK) and Alexa-488 and Alexa-647-conjugated secondary antibodies were bought from Life Technologies (Paisley, UK).

2.2. Animal and experimental design

All animal studies were approved by the Medical Research Council Harwell Institute Animal Welfare and Ethical Review Board, and all procedures were carried out within project license restrictions (PPL 30/3146) under the UK Animals (Scientific Procedures) Act 1986 and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Female C57BL6/N mice at 8 weeks old were fed either a control diet (C, 10% kCal fat) or a high-fat diet (HF, 60% kCal fat) for 6 weeks before pregnancy. They were then mated at 14 weeks of age with chow-fed males and kept on their respective diets during pregnancy (3 weeks) and lactation (4 weeks), totalling 13 weeks of dietary intervention on the dams. Upon weaning at 4 weeks, male offspring were randomly assigned by a technician, a control or high-fat diet for 26 weeks. Female offspring was used for a different study. Therefore, there were 4 experimental groups: C-fed dam and C-fed offspring (C/C), C-fed dam and HF-fed offspring (C/HF), HF/C and HF/HF. There were 3 distinct litters for HF/C and HF/HF and 4 for C/HF and C/C. 11 dams were used to produce different offspring groups. Throughout dietary intervention, mice were weighed weekly, metabolic cage analysis performed at 22 weeks whilst body composition and intraperitoneal glucose tolerance test (ipGTT) were measured at 26 weeks of age. At 30 weeks of age, blood was collected through retro-orbital sinus with mice under terminal anaesthesia, which consisted of inhalation of 5% isoflurane until full anaesthesia was achieved, determined by loss of pedal reflex. Death was confirmed by cessation of the circulation and neck dislocation. For serological analysis, blood was allowed to clot, then centrifuged at 3000 x g for 3 min and sera frozen at -80 °C until analysis. For platelet studies, blood was collected in 1:9 v/v acid citrate dextrose (ACD: 2.5% sodium citrate, 2% D-glucose, and 1.5% citric acid) and analysed after 1 hour of resting. A schematic diagram of the animal model can be found in Supplementary Fig. 1.

2.3. Serological analyses and full blood count

All samples for serological analyses and full blood count were processed by the MRC Clinical Chemistry core facility, MRC Harwell Institute (Oxfordshire, UK) using an AU680 Clinical Chemistry Analyser (Beckman Coulter, High Wycombe, UK) and performed as per manufacturer (Addenbrookes Hospital, Cambridge, UK). Full blood count was determined using mice-specific settings on an Advia 2120
dedicated veterinary blood counter (Siemens Healthcare, Surrey, UK). TyG index was calculated as: Ln [triglyceridaemia (mg/dL) x glycaemia (mg/dL)]/2.

2.4. Body composition analysis

Body composition was measured at 26 weeks of age by nuclear magnetic resonance (EchoMRI™, Houston, Texas, USA), which determined total body fat, lean mass and free water in grams. The percentage of each component was then calculated based on the total body weight of the animal.

2.5. Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance tests were performed at 26 weeks following a 16 hour fast. Specifically, fasted mice received an i.p. administration of 2 g/kg glucose and blood sampled under a local anesthetic at 0 min (baseline), 15, 30, 60 and 120 min post glucose injection. Whole blood glucose was measured using an AlphaTRAK meter and test strips (Abbott Animal Health, UK).

2.6. Indirect calorimetry

Indirect calorimetry was performed in 22 weeks old mice using a TSE PhenoMaster system (TSE Systems GmbH, Hamburg, Germany). Animals were individually placed in metabolic cages for 1 hour to acclimatize. Measurements started at 15:00 and finished at 11:00 of the next day. VO\textsubscript{2}, VCO\textsubscript{2}, respiratory exchange rate (RER), energy expenditure rate and locomotor activity were measured every 15 minutes under constant temperature of 20 °C.

2.7. Platelet activation and membrane receptor studies

Blood was collected through retro-orbital sinus in 1:9 v/v ACD tubes and centrifuged at 203 x g for 8 minutes to separate the platelet-rich plasma (PRP). PRP was then incubated in a 96-well plate with or without NO donor PAPA-NONOate at 100 µM for 10 minutes prior to addition of agonists ADP, CRP or thrombin at specified concentrations. FITC-labelled fibrinogen was incubated for 30 minutes in the dark followed by dilution (25X) with Tyrodes-HEPES buffer (134 mM NaCl, 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 12 mM NaHCO\textsubscript{3} 5 mM glucose, 0.34 mM Na2HPO\textsubscript{4}, 2.9 mM KCl and 1 mM MgCl\textsubscript{2}, pH 7.3). In order to measure platelet membrane receptor expression, whole blood was incubated with antibodies specific to membrane receptors or control IgG at the concentrations specified by the manufacturer for 30 minutes in the dark. In both assays, events were acquired using a BD Accuri C6 Plus flow cytometer (BD Biosciences, Oxford, UK) and platelets gated by forward and side scatter.

2.8. Platelet spreading

PRP was supplemented with 1.25 µg/mL PGI\textsubscript{2} and further centrifuged at 1,028 x g for 5 minutes and washed platelets (WP) pellet resuspended in Tyrodes-HEPES buffer. WP (2 x 10\textsuperscript{7} platelets/mL) were added to a coverslip coated with 100 µg/mL collagen and left to adhere for 45 minutes at 37 °C. Non-adherent platelets were washed off with PBS, and adherent platelets fixed with 0.2% paraformaldehyde, permeabilized with Triton-X 0.01% v/v for 10 minutes and subsequently stained with Alexa Fluor 488-
conjugated phalloidin (1:1000 v/v) for 1 hour in the dark at room temperature. Images were acquired using a Nikon A1-R Confocal microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands).

2.9. Reactive oxygen species (ROS) measurement

Whole blood (WB) was incubated with 20 µM ROS-detecting fluorescent dye DCFDA for 30 minutes in the dark and events acquired using a BD Accuri C6 Plus flow cytometer. Platelet and red blood cells populations were separated by forward and side scatter.

2.10. Immunoblotting

WP (2 × 10^8 platelets/mL) incubated with or without PAPA-NONOate at 100 µM for 10 minutes prior to addition of 1 µg/mL CRP. Reduced laemmli buffer was added 90 seconds after CRP addition to lyse platelets and stop reaction. This solution was heated for 10 minutes and processed exactly as previously described [16].

2.11. Statistical analysis

Statistical analyses were run in GraphPad Prism 8.0 software (GraphPad Software, San Diego, USA). Quantitative results in figures and tables were expressed as mean ± SEM and individual values. Overall, n = 17 for C/C, n = 10 for C/HF, n = 16 for HF/C and n = 13 for HF/HF. However, n number varied across assays. For metabolic and phenotypic studies n = 10–17 per group except for indirect calorimetry experiments, which had n = 3–8 due to low availability of metabolic cages and time-constraints. Functional platelet studies had n = 5–13 and immunoblots had n = 3–7 due to high blood volume needed per animal to perform all experiments. Outliers were identified and excluded using ROUT method. Data were considered of normal distribution due to sample size. Equal variance (sphericity) was not assumed since samples were independent and analysed through unpaired two-way ANOVA for bar graphs with Dunnett’s post-test to test differences vs C/C. Overall effect of maternal, offspring or the interaction of these are presented and interpreted throughout text. Data in XY graphs were assessed by repeated measures two-way ANOVA and Dunnett’s as post-test with level of significance of 5%.

3. Results

3.1. Maternal high-fat diet programmed phenotypic and metabolic parameters differently in lean and obese offspring

Several aspects of the metabolic phenotype as well as serum biochemistry were assessed in 30 weeks old male mice (Table 1). It was evident that both offspring HF groups presented significant increases in body weight, fat mass, total cholesterol, fasting glucose, TyG Index which is a surrogate for insulin resistance, as well as HDL and LDL levels when compared to offspring C groups. Interestingly, HF/C
animals were lighter and had increased lean mass as percentage of body weight when compared to C/C. HF/HF, but not C/HF, showed increased serum levels of triglycerides when compared to C/C group. Lack of interaction between maternal and offspring diet indicates an additive effect of these dietary interventions leads to elevated serum triglyceride levels.
Table 1
Maternal and offspring high-fat diet have additive effects on triglycerides and free fatty acid levels

|                          | C/C       | C/HF      | HF/C      | HF/HF     | MD       | OD       | MD x OD  |
|--------------------------|-----------|-----------|-----------|-----------|----------|----------|----------|
|                          | n = 17    | n = 10    | n = 16    | n = 13    |          |          |          |
| p-values                 |           |           |           |           | 0.0312   | < 0.0001 | 0.3205   |
| Final weight (g)         | 35.51 ± 1.043 | 51.21 ± 1.174* | 32.17 ± 1.035* | 49.96 ± 0.6757* |          |          |          |
| Lean weight (%)          | 72.34 ± 1.878 | 57.58 ± 1.279* | 79.07 ± 1.845* | 56.98 ± 1.273* |          |          |          |
| Fat mass (%)             | 27.9 ± 2.852 | 48.01 ± 1.182* | 26.55 ± 1.697 | 48.02 ± 1.203* | 0.6296   | < 0.0001 | 0.6253   |
| Total Cholesterol (mmol/L)| 3.991 ± 0.2509 | 6.809 ± 0.5478* | 3.653 ± 0.1479 | 6.928 ± 0.2402* | 0.7154   | < 0.0001 | 0.4472   |
| HDL (mmol/L)             | 2.439 ± 0.1574 | 3.839 ± 0.26*      | 2.254 ± 0.09727 | 3.858 ± 0.09275* | 0.6031   | < 0.0001 | 0.5217   |
| LDL (mmol/L)             | 1.12 ± 0.0593   | 1.97 ± 0.1881*      | 0.8256 ± 0.07187 | 1.768 ± 0.1241* | 0.0240   | < 0.0001 | 0.6650   |
| Triglycerides (mmol/L)   | 1.22 ± 0.06943 | 1.418 ± 0.1056       | 1.283 ± 0.1051 | 1.661 ± 0.169* | 0.1940   | 0.0166   | 0.4432   |
| Free fatty acids (mmol/L)| 0.6994 ± 0.03459 | 0.881 ± 0.0394*     | 0.7888 ± 0.04977 | 0.9338 ± 0.0681* | 0.1657   | 0.0021   | 0.7197   |
| Fasting glucose (mmol/L) | 7.435 ± 0.3304 | 11.21 ± 0.3719*     | 6.613 ± 0.2567 | 9.717 ± 0.5893* | 0.0049   | < 0.0001 | 0.3985   |

Data presented as mean ± SEM. N = 10–17 animals for each group. Groups were analyzed by Two-way ANOVA and Dunnet post-test for multiple comparisons vs C/C. * p < 0.05 vs C/C.

C/C: dam and offspring fed chow; C/HF: dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high fat diet. MD: Maternal diet. OD: Offspring diet.


|        | C/C   | C/HF  | HF/C  | HF/HF | MD   | OD   | MD x OD |
|--------|-------|-------|-------|-------|------|------|---------|
| TyG index | 8.842 ± 0.08041 | 9.419 ± 0.08766* | 8.756 ± 0.08764 | 9.378 ± 0.1141* | 0.5063 | < 0.0001 | 0.8101 |
|        |       |       |       |       |      |      |         |

Data presented as mean ± SEM. N = 10–17 animals for each group. Groups were analyzed by Two-way ANOVA and Dunnet post-test for multiple comparisons vs C/C. * p < 0.05 vs C/C.

C/C. dam and offspring fed chow; C/HF. dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high fat diet. MD: Maternal diet. OD: Offspring diet.

All groups were also assessed for their body weight growth, glucose homeostasis and indirect calorimetry to further characterize the model (Fig. 1). Both HF offspring groups presented increased body weight compared to C/C. HF/HF animals were consistently heavier than C/C starting at 7 weeks (p < 0.01), whereas C/HF started at 8 weeks of age (Fig. 1A). Of note, HF/C mice were lighter than C/C over the last 4 weeks of the study (Fig. 1A), in agreement with the increased lean weight described in Table 1. To assess glucose homeostasis of these animals, ipGTT was performed and evidenced that both offspring HF groups were glucose intolerant, whereas HF/C showed improved glucose homeostasis compared to C/C (Fig. 1B). Overall, both HF/C and HF/HF presented severe metabolic dysfunction since these mice were obese, had increased accumulated fat, were insulin resistant and glucose intolerant.

Indirect calorimetry was measured from individual animals placed in a metabolic cage. Due to restricted cage availability, sample size was reduced. Offspring HF groups showed a lower RER coupled with decreased energy expenditure, suggesting increased burning of fat and diminished metabolic activity respectively (Fig. 1C – G), which would contribute to the other metabolic dysfunctions present in these animals. Surprisingly, HF/C also showed lower RER compared to C/C, though not as pronounced as offspring HF groups. Total ambulatory activity was not changed across groups, likely due to the high variability of the assay (Fig. 1H). In summary, maternal HF programmed the offspring differently, depending on the diet given to the offspring: if given a high-fat diet, offspring presented more severe metabolic dysfunctions in terms of triglycerides levels; if given standard chow, offspring burned more fat, were lighter and had improved glucose homeostasis.

### 3.2. Maternal and offspring high-fat diet ingestion led to increased platelet volume

It has been shown that increased mean platelet volume (MPV) is correlated with cardiovascular events [17], therefore full blood count was performed (Table 2). There was a 30% decrease in total leukocytes on the HF/C group compared to C/C, which suggests leukocytes could be programmed by maternal high-fat diet ingestion, although there was no overall effect (two-way ANOVA, p = 0.06). HF/HF animals had a significant 0.7 fL increase in MPV when compared to C/C. Indeed, MPV was significantly increased by
maternal (two-way ANOVA, maternal diet effect p = 0.01) and offspring (p = 0.02) high-fat diet. The effect of maternal and offspring high-fat diet were independent since there was no interaction, indicating an additive effect between maternal and offspring diet on platelet size.
Table 2
Maternal and offspring high-fat diet leads to increased platelet volume

|                      | C/C     | C/HF  | HF/C   | HF/HF  | MD       | OD       | MD x OD |
|----------------------|---------|-------|--------|--------|----------|----------|---------|
| n                    | 17      | 10    | 16     | 13     |          |          |         |
| **P-values**         |         |       |        |        |          |          |         |
| **Red blood cells**  |         |       |        |        |          |          |         |
| Hematocrit (L/L)     | 0.4831  | 0.478 | 0.5003 | 0.4927 | 0.0891   | 0.4948   | 0.8920  |
| ± 0.004511           | ± 0.01351| ± 0.00524| ± 0.01322|         |          |          |         |
| RBC (x10^6 cells/µL) | 9.382   | 9.462 | 9.715  | 9.612  | 0.2199   | 0.9538   | 0.6395  |
| ± 0.1229            | ± 0.2112| ± 0.1014| ± 0.3037|         |          |          |         |
| Haemoglobin (g/dL)   | 13.81   | 12.78 | 13.69  | 13.41  | 0.4502   | 0.0568   | 0.2666  |
| ± 0.1303            | ± 0.6418| ± 0.1818| ± 0.3974|         |          |          |         |
| MCV (fL)             | 51.05   | 51.24 | 51.39  | 51.12  | 0.7811   | 0.9235   | 0.5413  |
| ± 0.2816            | ± 0.4708| ± 0.4028| ± 0.3406|         |          |          |         |
| MCH (pg)             | 14.38   | **13.14** | 13.95  | 14.08  | 0.3892   | 0.0643   | **0.0222** |
| ± 0.1657            | ± 0.5818*| ± 0.2332| ± 0.2124|         |          |          |         |
| MCHC (g/dL)          | 28.19   | **25.94** | 27.35  | 27.42  | 0.4627   | **0.0136** | **0.0091** |
| ± 0.2743            | ± **0.8672** | ± 0.2861| ± 0.2897|         |          |          |         |
| CHCM (g/dL)          | 26.29   | **25.12** | 25.73  | 25.63  | 0.9317   | **0.0396** | 0.0800  |
| ± 0.2057            | ± **0.6209** | ± 0.2369| ± 0.184|         |          |          |         |
| RDW (%)              | 13.57   | **15.29** | 13.08  | 13.97  | **0.0193** | **0.0011** | 0.2699  |
| ± 0.2715            | ± **0.8498** | ± 0.1595| ± 0.1305|         |          |          |         |

White blood cells

Data presented as mean ± SEM. N = 10–17 animals for each group. Groups were analyzed by Two-way ANOVA and Dunnet post-test for multiple comparisons vs C/C. * p < 0.05 vs C/C.

C/C. dam and offspring fed chow; C/HF. dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high fat diet. MD: Maternal diet. OD: Offspring diet.
|                          | C/C n = 17 | C/HF n = 10 | HF/C n = 16 | HF/HF n = 13 | MD | OD | MD x OD |
|--------------------------|------------|-------------|-------------|--------------|-----|----|--------|
| **P-values**             |            |             |             |              |     |    |        |
| **Leukocytes (x10^3 cells/μL)** |            |             |             |              |     |    |        |
|                           | 6.099 ± 0.615 | 5.297 ± 0.5024 | 4.322 ± 0.3762* | 5.189 ± 0.1749 | 0.0685 | 0.9493 | 0.1055 |
| **Neutrophils (x10^3 cells/μL)** | 0.5908 ± 0.06151 | 0.524 ± 0.05959 | 0.5344 ± 0.05547 | 0.5038 ± 0.02379 | 0.4778 | 0.3677 | 0.7363 |
| **Neutrophils (%)**      | 12.59 ± 1.06 | 9.456 ± 1.143 | 11.56 ± 0.7853 | 8.946 ± 0.4991* | 0.3980 | 0.0026 | 0.7744 |
| **Lymphocytes (x10^3 cells/μL)** | 4.086 ± 0.2678 | 4.256 ± 0.3835 | 3.673 ± 0.3631 | 4.853 ± 0.3103 | 0.7843 | **0.0491** | 0.1375 |
| **Lymphocytes (%)**      | 80.19 ± 1.371 | 83.63 ± 1.674 | 82.09 ± 0.9376 | 84.00 ± 0.9541 | 0.3583 | **0.0340** | 0.5327 |
| **Monocytes (x10^3 cells/μL)** | 0.19 ± 0.01766 | 0.167 ± 0.02813 | 0.1013 ± 0.01125* | 0.19 ± 0.01649 | 0.0781 | 0.0781 | **0.0036** |
| **Monocytes (%)**        | 3.194 ± 0.2094 | 3.18 ± 0.4864 | 2.294 ± 0.2305 | 3.354 ± 0.3096 | 0.2246 | 0.0827 | 0.0750 |
| **Eosinophils (x10^3 cells/μL)** | 0.1031 ± 0.013 | 0.095 ± 0.01721 | 0.1388 ± 0.02862 | 0.09615 ± 0.01083 | 0.3723 | 0.2202 | 0.4029 |
| **Eosinophils (%)**      | 1.813 ± 0.2658 | 1.82 ± 0.2913 | 2.619 ± 0.3764 | 1.754 ± 0.2533 | 0.2509 | 0.1845 | 0.1770 |

**Platelets**

Data presented as mean ± SEM. N = 10 – 17 animals for each group. Groups were analyzed by Two-way ANOVA and Dunnet post-test for multiple comparisons vs C/C. * p < 0.05 vs C/C.

C/C: dam and offspring fed chow; C/HF: dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high fat diet. MD: Maternal diet. OD: Offspring diet.
|                          | C/C  | C/HF | HF/C | HF/HF | MD    | OD    | MD x OD |
|--------------------------|------|------|------|-------|-------|-------|---------|
| Number                   | n = 17 | n = 10 | n = 16 | n = 13 |       |       |         |
| Platelet count (x10³ cells/µL) | 1530 ± 82.88 | 1366 ± 121.5 | 1468 ± 116.5 | 1308 ± 73.33 | 0.5602 | 0.1201 | 0.9865 |
| MPV (fL)                 | 8.112 ± 0.1725 | 8.46 ± 0.1176 | 8.513 ± 0.111 | 8.8 ± 0.105* | 0.0117 | 0.0293 | 0.8312 |
| PDW (%)                  | 48.04 ± 0.899 | 49.71 ± 0.941 | 48.00 ± 0.6116 | 51.88 ± 0.9178* | 0.2316 | 0.0027 | 0.2167 |
| PCT (%)                  | 1.245 ± 0.07551 | 1.158 ± 0.1063 | 1.253 ± 0.1008 | 1.151 ± 0.06764 | 0.9999 | 0.3014 | 0.9368 |

Data presented as mean ± SEM. N = 10–17 animals for each group. Groups were analyzed by Two-way ANOVA and Dunnet post-test for multiple comparisons vs C/C. * p < 0.05 vs C/C.

C/C: dam and offspring fed chow; C/HF: dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high fat diet. MD: Maternal diet. OD: Offspring diet.

### 3.3. Maternal high-fat diet ingestion increases offspring platelet adhesion and spreading to collagen

Upon vascular injury, platelets are exposed and adhere to collagen. Therefore, we assessed the ability of platelets from all groups to adhere and spread to immobilized collagen and undergo cytoskeletal reorganisation, referred to as spreading (Fig. 2). When compared to C/C, platelets from HF/HF group exhibited a 9 and 1.5 fold increase in platelet adherence and spreading, respectively. This effect was not seen in C/HF, whilst there was an increase in adherence in the HF/C group. The overall effect of maternal high-fat diet ingestion led to increased adhesion (two-way ANOVA, p < 0.0001) and spreading (p = 0.0012) independent of offspring diet, since there was no interaction between maternal and offspring diet.

### 3.4. High-fat diet ingestion in both offspring and dams let to platelet hyperactivity and decreased surface expression levels of collagen receptors

Given the severe metabolic dysfunction, increased adhesive potential and increased platelet size found in HF/HF animals, we assessed platelet activation by measuring fibrinogen binding to platelet integrin αIIbβ3 using FITC-conjugated fibrinogen (Fig. 3). PRP was isolated from whole blood and stimulated with...
different concentrations of the platelet activators ADP, CRP (a GPVI collagen receptor agonist) or thrombin. Platelets of HF/HF animals bound to fibrinogen 3x more than C/C when stimulated with ADP and 2x more when stimulated with CRP (Fig. 3A and B), however, for ADP there was an interaction between maternal and offspring diet. This indicates that the increase in fibrinogen binding seen in HF/HF is dependent on both maternal and offspring high-fat diet ingestion, thus not being an effect solely due to maternal diet. Interestingly, there was no increase when platelets were stimulated with thrombin (Fig. 3C), suggesting that rises in fibrinogen binding are specific to some agonists and are unlikely to be due to increased MPV in HF/HF. The abovementioned effects were similar when different doses of agonists were used or when platelets were resting (Supplementary Fig. 2).

Surface expression levels of key platelet receptors were measured through flow cytometry. Both offspring HF groups showed decreased levels of GPVI and integrin $\alpha_2$ compared to C/C (Fig. 4A and B), indicating increased spreading on collagen and CRP-induced activation observed in HF/HF platelets was not due to upregulated GPVI surface expression. Interestingly, maternal obesity resulted in decreased expression levels of collagen receptors, suggesting possible epigenetic regulation. There were no differences between groups on surface levels of oxidized LDL receptor CD36 or von willebrand factor receptor CD42b (Fig. 4C and D), despite overall effects of maternal and offspring diets.

3.5. Both maternal and offspring high-fat diet induce increased oxidative stress in whole blood

We measured unspecific ROS production in whole blood to test the hypothesis that oxidative stress, often associated with metabolic dysfunction, could contribute to the observed platelet hyperreactivity (Figs. 2 and 3). Both maternal and offspring HF groups presented an increase in oxidative stress of $\sim$ 100% in platelets and $\sim$ 50% in red blood cells when compared to C/C. These effects were dependent on both maternal and offspring dietary intervention, since there was interaction of terms (Fig. 5A and B). This increase in ROS may contribute to the increase platelet functional effects observed so far, although further experiments are needed to show a causal link.

3.6. Increased signalling in platelets from HF/HF animals

Following the observation that GPVI levels were reduced, but GPVI-mediated platelet function was increased in HF-HF mice, we stimulated platelets with CRP for 90 seconds and performed immunoblots to characterize the effects on intracellular signalling downstream of GPVI (Fig. 6). Compared to C/C, platelets from HF/HF animals presented increased Akt phosphorylation at Ser473 (Fig. 6A), as well as increased tyrosine (4G10 antibody, Fig. 6B) and PKC substrate phosphorylation (Fig. 6C). Out of these, maternal diet led to increased Akt phosphorylation independent of offspring diet (two-way ANOVA, $p = 0.02$), since there was no interaction. The responses to nitric oxide donor PAPA-NONOate were also assessed. VASP phosphorylation was unchanged across groups, although C/ HF mice presented slightly increased PAPA-NONOate inhibition in ADP-stimulated platelets and slightly decreased inhibition in CRP-
stimulated ones (Supplementary Fig. 3). These data indicate that the hyperactivation of HF/HF platelets were associated to increased signalling downstream of GPVI.

4. Discussion

This is the first study to describe the impacts of maternal metabolic dysfunction on the platelet activity of the offspring. We show that HF animals born from HF dams presented metabolic dysfunction, with higher serum levels of triglycerides, as well as developed heavier body weight earlier than HF offspring whose mothers were lean (p < 0.01 at week 7). Maternal obesity led to increased spreading over collagen, decreased surface expression of collagen receptors and increased oxidative stress. Platelets from HF/HF animals were larger, hyper-reactive and with increased signalling, suggesting multiple mechanisms leading to increased platelet activation. Therefore, we suggest a novel ‘double-hit’ effect of maternal and offspring high-fat diet ingestion that causes platelet hyperactivation in the offspring.

To begin with, we assessed metabolic and phenotypic parameters to characterize our model. Our data show that maternal high-fat diet decreased body weight of chow-fed offspring after 18 weeks of age, when HF/C animals began to gain less weight than C/C animals. The majority of studies assessing effects of maternal obesity on offspring have used offspring at a younger age and showed increased or unchanged body weight and adiposity in chow-fed male offspring born to obese dams [18, 19]. Similar to our data, Blackmore et al [20] showed male mice born to obese dams to have a trend towards lighter body weight and improved metabolic parameters at 12 and 8 weeks of age, respectively. They have also described an increased sympathetic activity in the heart of these mice, compared to mice born to lean dams. It is possible that maternal obesity may programme the offspring to adapt to a fat-rich environment through higher adrenergic discharge, potentially explaining the increased tendency to burn fat in HF/C offspring. This may represent an adaptive response to perceived nutritional stress such as those termed ‘predictive adaptive responses’ [21], however it is not clear why these potential adaptive changes occur at a delayed point during the life-course of the offspring. Further work is necessary to examine this potential adaptive response and indeed to confirm whether higher sympathetic activity may account for the effects on body weight.

Our results showed that if mice born to HF dams were weaned onto an obesogenic diet, these mice displayed increased body weight, adiposity and serum cholesterol levels as well as glucose intolerance, decreased RER and energy expenditure compared C/C mice. Increased body weight was comparable between HF/HF and C/HF, similar to a previous report by Loche et al [22]. The effects of maternal and offspring high-fat diet were additive on serum triglycerides levels. This is in agreement with previous reports describing a consistent [23] or a trend [18] towards increased serum levels of triglycerides in similar murine models, albeit using younger mice. Increased triglyceridaemia have been shown to aggravate other metabolic functions, such as insulin resistance (for review see [24]). Likewise, hypertriglyceridaemia was correlated with increased platelet activity both after acute intralipid injection [25] and in chronic obese patients [26]. Therefore, it is possible that the additive effect of maternal and
offspring obesity on serum triglycerides may be correlated with altered platelet function and increased cardiovascular risk.

In agreement with increased serum triglycerides levels, HF/HF mice presented altered platelet size and function. These mice had larger platelets with enhanced spreading on collagen as well as activation induced by two agonists that act through distinct mechanisms. Interestingly, maternal or offspring high-fat diet ingestion had an overall effect of increasing MPV in offspring. In contrast, only maternal obesity led to increased platelet spreading over collagen, suggesting that increased platelet size does not fully explain this result. In line with this, platelets from HF/HF mice were hyperreactive to ADP and CRP, but not to thrombin. Therefore, agonist-specific effects reiterates that increased platelet function consequent of maternal high-fat diet ingestion is not fully explained by increased MPV alone.

The fact that only HF/HF mice presented increased platelet activation suggests a previously undescribed 'double-hit' effect of maternal and offspring obesity, in which both insults are needed to alter platelet function. This is in line with a previous epidemiological report describing an association between maternal obesity and premature mortality from cardiovascular events [27], to which platelets are intrinsically related [28]. Therefore, it is possible that the platelet hyperactivation herein observed in HF offspring born to HF dams can be a key pathophysiological component of the cardiovascular consequences of maternal obesity described in humans. Future research will explore epigenetic changes in platelets and megakaryocytes to better understand this phenomenon.

Platelet GPVI and integrin α2 expression was decreased due to maternal high-fat diet ingestion. We believe these alterations could be a consequence of shedding, in case of GPVI [29] or epigenetic changes in case of integrin α2. Considering that all HF groups presented increased levels of circulating ROS and that GPVI activation is both cause and consequence of increased ROS production in platelets [30], it is possible that oxidative stress interferes with GPVI expression and response to CRP. This, however, may not be the only pathway involved, given that HF/C and C/HF mice had normal platelet function, highlighting the potential for developmental programming at the epigenetic level based on the metabolic profile of the mother.

Barrachina et al have recently shown that platelets from obese individuals are hyperresponsive to CRP and that they express higher levels of GPVI when compared to non-obese individuals [31]. Using non-human primates, Arthur et al [32] demonstrated that platelets from diabetic monkeys produce more ROS and are more responsive to CRP despite having unaltered levels of GPVI. There is a lack of reports assessing platelet GPVI levels in obesity and, although not directly comparable, the abovementioned studies flag the importance of the GPVI signalling pathway to the platelet dysfunction observed metabolic diseases. We argue that this might also be true for the consequences of maternal obesity on the platelet hyperactivation seen in obese offspring.

Besides differently expressed receptor levels, platelets from HF/HF mice also showed increased PKC substrate, total tyrosine and Akt phosphorylation; an effect not seen in other groups. Although we only studied these following GPVI activation, they are all key signalling events common to a range of agonists
(for review, see [33]), therefore it will be interesting to extend this work to identify whether other signalling pathways are affected or whether this is a GPVI-specific effect. Moreover, it has been shown that platelets from obese individuals are less sensitive to inhibitory molecules such as NO [34]. Our data do not support differences in NO sensitivities, suggesting that metabolic dysfunction in both maternal and offspring favours platelet dysfunction through higher sensitivity to stimulatory signals rather than lower responsiveness to inhibitory ones. Nevertheless, this should be further explored in the future with the use of other inhibitors, such as aspirin and PGI₂.

We acknowledge several limitations in this study. As this is the first report on the effect of maternal obesity on platelet function, we did not exhaust all aspects of platelet function. Future research could use different approaches, such as: platelet aggregation, calcium mobilization, and thrombus formation *in vitro*. Likewise, epigenetic changes due to maternal obesity were not explored and could provide interesting insights on the precise mechanism of the phenotype herein observed. We recognize that data *in vitro* do not always translate in functional consequences *in vivo* and therefore encourage future reports to assess the effects of maternal obesity on thrombus formation *in vivo* and link animal data with human studies to support or discard our hypothesis. Finally, we believe that future studies should address sex-specific effects of maternal metabolic dysfunction on platelet function.

5. Conclusion

To summarize, we propose that platelets can be programmed by metabolic dysfunction in mothers and that there is a 'double-hit' effect that leads to platelet hyperactivity. The molecular mechanisms involved decreased GPVI expression, increased ROS production and enhanced phosphorylation levels of key stimulatory signalling proteins. Also, maternal high-fat diet ingestion per se seemed to induce a pro-adaptive metabolic response in chow-fed offspring, since these animals were leaner and tended to burn more fat than their counterparts born to lean dams. This should be further explored due to the lack of literature assessing offspring as old as those used in this report. These findings shed light on possible pathophysiological explanations to the increased risk of cardiovascular events in individuals born to mothers with metabolic dysfunction and add yet another layer of evidence to the deleterious effects of maternal obesity to the health of their offspring.

6. List Of Abbreviations

AKT – Protein Kinase B
CRP - Collagen-related Peptide
DCFDA – 2′,7′-Dichlorofluorescin diacetate
FFA – Free Fatty Acids
GPVI – Glycoprotein VI
7. Declarations

Ethics approval

All animal studies were approved by the Medical Research Council Harwell Institute Animal Welfare and Ethical Review Board, and all procedures were carried out within project license restrictions (PPL 30/3146) under the UK Animals (Scientific Procedures) Act 1986 and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.
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Author contributions

RSG designed the study, performed experiments, analysed data and drafted the manuscript. AJU and AB helped designing the study, performed experiments and analysed data. TS performed experiments. JMG helped designing the study, discussed data and reviewed the manuscript. CH designed the study, performed experiments, discussed data and supervised drafting the manuscript. DS, RDC, SW and MS designed and supervised the animal model and contributed to drafting of the manuscript. Authors reviewed the final draft and approved the final version of the manuscript.

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Figure 1

Metabolic effects of maternal obesity depend on offspring diet. (A) Growth curve with weekly weight measures. (B) Intraperitoneal glucose tolerance test (ipGTT) with glucose measured at 0, 15, 30, 60 and 120 min after glucose injection. (C) Respiratory exchange rate (RER), (D) maximum rate of oxygen consumption (VO2), (E) maximum rate of carbon dioxide consumption (VCO2), (F) energy expenditure of total weight, (G) energy expenditure of lean weight and (H) total ambulatory activity were measured in individual mice placed in a metabolic cage for 20 hours. C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high-fat diet. For (A) and (B), n=10-17 mice per group. For (C–H), n=4 for C/C, n=3 for C/HF, n=8 for HF/C and n=8 for HF/HF. Data on graphs show mean ± SEM. Data analysed by repeated measures two-way ANOVA with Dunnett’s multiple comparisons test vs C/C. *p<0.05 and colour of stars indicate significance of the group against C/C.
Maternal high-fat diet increased platelet spreading over collagen. WP (2 x 10^7 platelets/mL) was left to adhere to coverslips coated with collagen and platelets fluorescently labelled for visualization. (A) Representative images of platelet spreading of each group. (B) Quantification of platelet adherence as number of platelets per field. (C) Platelet spreading as the total fluorescence of the field divided by the number of platelets. C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high-fat diet. N=5-13 mice per group. Graphs show mean ± SEM as well as individual values. * p<0.05 vs C/C. Data analysed by two-way ANOVA with Dunnett's multiple comparisons test vs C/C. The overall effects of maternal and offspring diet are reported where significant.
Maternal and offspring high-fat diet ingestion resulted in platelet hyperactivation. Platelet-rich plasma (PRP) was stimulated with 10 μM ADP (A), 3 μg/mL CRP (B) or 0.1 U/mL Thrombin (C) and FITC-conjugated fibrinogen binding measured through flow cytometry. C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high-fat diet. N=5-13 mice per group. Graphs show mean ± SEM as well as individual values. **** p<0.0001 vs C/C. Data analysed by two-way ANOVA with Dunnett’s multiple comparisons test vs C/C. The overall effects of maternal and offspring diet are reported where significant.
Maternal and offspring high-fat diet ingestion decreased surface expression levels of collagen receptors. Platelet-rich plasma (PRP) was incubated with antibodies for GPVI (A), β2 integrin (B), CD36 (C) and Gp1bα (D) receptors and measured using flow cytometry. C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high-fat diet. N=5-13 mice per group. Graphs show mean ± SEM as well as individual values. **** p<0.0001 vs C/C. Data analysed by two-way ANOVA with Dunnett’s multiple comparisons test vs C/C. The overall effects of maternal and offspring diet are reported where significant.
Maternal or offspring high-fat diet induced increased oxidative stress in whole blood. Whole blood was diluted 25 times with Tyrodes-HEPES buffer and incubated with 20 µM ROS-detecting fluorescent dye DCFDA. Events acquired using a flow cytometer and platelet (A) or red blood cells (RBC, B) populations separated by forward and side scatter. C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high-fat diet. N=10-17 mice per group. Graphs show mean ± SEM as well as individual values. * p<0.05 vs C/C; ** p<0.01 vs C/C; **** p<0.0001 vs C/C. Data analysed by two-way ANOVA with Dunnett’s multiple comparisons test vs C/C. The overall effects of maternal and offspring diet are reported where significant.
Figure 6

Maternal and offspring high-fat diet ingestion enhanced platelet signalling. Washed platelets (WP) were stimulated with CRP for 90 seconds and lysed. Representative immunoblot and summarized data are shown for phosphorylation of Akt at Ser473 (A), 4G10 total tyrosine phosphorylation (B) and PKC substrate serine phosphorylation (C). C/C, dam and offspring fed chow; C/HF dam fed chow and offspring fed high-fat diet; HF/C dam fed high-fat diet and offspring fed chow; HF/HF dam and offspring fed high-fat diet. N=3-7 mice per group. Graphs show mean ± SEM as well as individual values. * p<0.05 vs C/C. Data analysed by two-way ANOVA with Dunnett’s multiple comparisons test vs C/C. The overall effects of maternal and offspring diet are reported where significant.

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