Fetal Programming and Lactation: Modulating Gene Expression in Response to Undernutrition during Intrauterine Life

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Impact:

- Heart remodeling caused by fetal programming involves the alteration of several groups of genes and lactation period plays a key role in establishing gene expression modification.

- We could identify expression changes of relevant genes in cardiac tissue induced by undernutrition during fetal life.

- We expose the contribution of the lactation period in modulating the expression of Agt and Pparg, relevant genes associated with cardiac hypertrophy.

- This evidence reveals lactation as a crucial intervention window for preventing or countering fetal programming.

ABSTRACT

Background

Adverse environmental conditions during intrauterine life, known as fetal programming, significantly contribute to the development of various diseases in adulthood. Fetal programming induced by factors like maternal undernutrition leads to low birth weight and increases the risk of cardiometabolic diseases.

Methods

We studied a rat model of maternal undernutrition during gestation (MUN) to investigate gene expression changes in cardiac tissue using RNA-sequencing of day 0-1 litters. Moreover, we analyzed at weaning the impact of lactation through cross-fostering experiments on cardiac structure and function assessed by transthoracic echocardiography, and gene expression changes though qPCR.

Results

Our analysis allowed to identified specific genes with altered expression in MUN rats at birth. Two of these genes, *Agt* and *Pparg*, stand out for being associated with cardiac hypertrophy and fibrosis. At the end of the lactation period (day 21), MUN males, but not females, showed increased expression of *Agt* and left ventricular hypertrophy; MUN males and females showed decreased expression of *Pparg*,. Cross-fostering experiments revealed that lactation with control breastmilk modulated these expression changes and reduced cardiac hypertrophy in MUN males.

Conclusion

Our findings highlight the interplay between fetal programming, gene expression, and cardiac hypertrophy. This suggests lactation as a potential intervention window to prevent or counteract the effects of fetal programming on cardiometabolic diseases and holds promise in combating diseases caused by adverse intrauterine environments.

66 **INTRODUCTION**

67 Adverse environmental conditions during intrauterine life play a critical role in shaping
68 short-term outcomes and significantly contribute to the development of various diseases
69 in adulthood. This phenomenon is known as fetal programming, or Developmental
70 Origins of Health and Disease (DOHaD). Extensive epidemiological and animal studies
71 demonstrated that exposure to a sub-optimal fetal environment has a detrimental effect,
72 substantially increasing the risk of conditions such as diabetes mellitus, obesity,
73 hypertension, and kidney and heart diseases, among others [1–3].

74 Fetal programming may be induced by adverse conditions during gestation, such as
75 infection, hypoxia, maternal caloric deficiency, or excess, as well as several obstetric
76 complications, like gestational diabetes, hypertension and preeclampsia. Among the most
77 extensively studied stressors implicated in fetal programming in humans is maternal
78 undernutrition, which results in low birth weight, rising the risk of developing
79 cardiometabolic diseases [4–6]. These alterations have also been confirmed in animal
80 models, which are crucial for studying the mechanisms involved in this association. In a
81 rat model of maternal undernutrition during gestation (MUN), it has been previously
82 established the development of hypertension in the offspring during adult life,
83 accompanied by postnatal cardiac hypertrophy and oxidative stress, with males being
84 more affected [7,8]. Moreover, previous studies in MUN model carried out in our group have
85 gathered evidence supporting the involvement of the Renin-Angiotensin System (RAS)
86 in this process [9,10] as well as highlighting the significant role of the lactation period in
87 modulating the phenotype [11,12].

88 Epigenetic changes induced by an adverse intrauterine environment are implicated in fetal
89 programming [13–15], and can also account for sexual dimorphic responses by modulating
90 gene expression [16]. However, the specific genes that undergo upregulation or
91 downregulation in fetal tissues due to these epigenetic changes are still under
92 investigation. Postnatal life represents a second critical period that can determine the final
93 trajectory of an individual, either consolidating or counteracting prior modifications in
94 gene expression. Exposure to undernutrition during intrauterine life results in genetic
95 programming that prepares the fetus to cope up with nutrient scarcity. Nevertheless, if
96 there are changes in nutritional conditions following birth, the discrepancy between
97 predicted and actual postnatal environments may be key in the development of
98 cardiometabolic disorders [13]. Breastfeeding is a factor that offers protection against the
99 long-term development of cardiometabolic diseases [17,18]. Breastfeeding not only supplies

100 bioactive components, but also promotes a slower, more physiological growth pattern.
101 Conversely, accelerated growth during lactation in infants born at term or preterm (either
102 normal or low birth weight) contributes to the consolidation of fetal programming and the
103 subsequent development of cardiometabolic diseases [19]. In rats exposed to undernutrition
104 during the fetal period, we have demonstrated the importance of the lactation environment
105 and postnatal growth pattern, evidencing that a slower growth during postnatal life
106 counteracts the development of cardiovascular and adipose tissue hypertrophy [20].
107 To gain a better understanding of the gene expression changes induced by fetal program-
108 ming, which subsequently lead to cardiovascular alterations, and to assess the impact of
109 lactation in this process, we used the previously described MUN model. This model is
110 characterized by intrauterine growth restriction and postnatal catch-up growth [21,22], which
111 has been associated with cardiac hypertrophy [11,20]. The primary objectives of this study
112 were twofold: first, to identify the gene expression changes in cardiac tissue induced by
113 undernutrition during fetal life, and second, to investigate the contribution of the lactation
114 period in modulating the expression of relevant genes associated with cardiac hypertro-
115 phy.

116 **METHODS**

117 **Animal model and Cross-Fostering Protocol**

118 Sprague Dawley rats from the colony bred at the Animal House of Universidad Autónoma
119 de Madrid (ES-28079-0000097) were used. The experiments were conducted according
120 to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of
121 Health publication no. 85-23, revised in 1996), the Spanish legislation (RD 53/2013), and
122 the Directive 2010/63/EU. Experimental procedures were approved by the Ethics Review
123 Board of Universidad Autónoma de Madrid and the Regional Committee of Comunidad
124 Autónoma de Madrid (PROEX 19/04; approved on March 20th 2019).
125 We used a rat model of fetal programming induced by maternal undernutrition (MUN)
126 during the second part of gestation, as previously described [7]. Briefly, after observation
127 of sperm in the vaginal smear (gestation = day 0) the dam was allocated to either MUN
128 or the Control group. Control dams were fed *ad libitum* throughout pregnancy and
129 lactation; MUN dams were fed *ad libitum* from day 1–10, and with 50% of the averaged
130 control daily intake (previously established as 24 g/day) from day 11 to the end of
131 gestation, returning to *ad libitum* diet during lactation. All the rats were fed with a
132 EuroRodent breeding diet (5LF5; Labdiet, Madrid, Spain) containing 22.0% protein,
133 4.4% fat, 55.0% carbohydrates, 4.1% fiber, and 5.4% mineral. Water was provided *ad*

134 *libitum* through the study. We studied the offspring at two age points: at birth and at
135 weaning. After birth the pups were sexed and the litter was standardized to 12 individuals,
136 6 males, and 6 females if possible, using the remaining pups to obtain hearts for gene
137 analysis. Control and MUN litters were left with their mothers or Cross-fostered and
138 studied at the age of 21 days (weaning period). Some animals were left for later studies.
139 Cross fostering was performed as previously described [20]**.** Two dams were mated at the
140 same time and those with birth on the same day were Cross-fostered on postnatal day 1
141 by exchanging the 12 pups to the foster's dam cage with bedding from the original mother
142 to avoid rejection. Four experimental groups were studied Control-on-Control (C; n = 4
143 mothers), MUN-on-MUN (MUN; n = 4 mothers); Control-on-MUN (C-on-MUN, n =4
144 mothers), MUN-on-Control (MUN-on-C; n =4 mothers). The name of the groups refers
145 to "pup type–on–mother type".

146 **RNA-sequencing and differential expression analysis**

147 Three biological replicates of three litters from MUN and Control of day 0-1 male rats
148 were used for RNA extraction. Left ventricle tissue was dissected, and frozen and RNA
149 extraction performed with RNeasy Mini Kit (Qiagen, Hilden, Germany). Sample RNA
150 yield was measured with a NanoDrop, precipitated in ethanol, and then sent to The
151 Genomics Unit of the Madrid Science Park (Madrid, Spain) for library preparation and
152 sequencing. Yield was checked, upon receipt of each sample, by use of NanoDrop, Qubit
153 RNA Assay and Agilent Bioanalyzer. The samples were fragmented after RNA QC,
154 reverse transcribed with random primers, and barcode tagged. Sequencing was performed
155 by on the Illumina Hiseq 2500, in 1x75 bp single-read sequencing configuration (the
156 output was stored as FASTQ-files), which yielded 25–30 million reads/sample. The
157 FASTQ-files were aligned against to *Rattus norvegicus* genome Rnor_5.0 reference
158 genome (Baylor College of Medicine Human Genome Sequencing Center and the Rat
159 Genome Sequencing Consortium (RGSC)) and raw reads were normalized as reads per
160 kilobase-length of gene per million mapped sequence reads (RPKM). Raw RNA-Seq
161 reads were aligned using TopHat (version 2.1.1) [23] with default settings, and only
162 uniquely mapped reads were retained to compute the number of reads for exons and exon-
163 exon junctions in each sample using the Python package HTSeq. The package DESeq2
164 (Galaxy Version 2.11.40.2) [24] was used to formulate the counts of the reads that were
165 aligned to each isoform of each event and the differential expression of transcripts. The
166 Benjamini-Hochberg procedure was applied to calculate the adjusted p-values in the

167 likelihood ratio test [25]. Gene Ontology enrichment analysis (GO) was performed using
168 PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification
169 system for GO Biological process, GO Molecular function, and GO cellular component;
170 lowest annotation levels, Hypergeometric statistical test, FDR (False Discovery Rate) P-
171 value correction [26,27].

172 **QPCR**

173 RNA was extracted from the left ventricle of the heart of three to six male and female
174 littermates from each group, namely C-on-C (n = 6), MUN-on-MUN (n = 6), C-on-MUN
175 (n = 3), and MUN-on-C (n = 3), at day 0 and day 21 after birth. The heart tissues were
176 weighed, frozen, mechanically disrupted, and subjected to RNA extraction using the
177 RNeasy Mini Kit (Qiagen, Hilden, Germany). Subsequently, the sample RNA yield was
178 measured using a NanoDrop spectrophotometer. For complementary DNA (cDNA)
179 synthesis, the total RNA (0.15-0.2 μg per reaction) was used, and the RT High-Capacity
180 cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific Inc.)
181 with random primers was employed, following the manufacturer's protocol.
182 Quantitative PCR (qPCR) was conducted using the 7500 Fast Real-Time PCR System
183 (Applied Biosystems) with technical duplicates. The PCR Power SYBR Green PCR
184 Master Mix (Applied Biosystems) was utilized for the reaction.
185 To determine gene relative expression, normalization was performed against the
186 housekeeping gene *Gapdh* [28], and the experimental group "male 21 weeks C-on-C" was
187 used as the reference sample. The 2^(-ΔΔCT) formula was employed to calculate relative
188 expression values based on the Ct values obtained.
189 RT-qPCR primers sequences were as follows*: peroxisome proliferator-activated receptor*
190 *gamma* (*Pparg*)-Forward (FW) 5'- CCTGTTGACCCAGAGCATGG -3'; *Pparg*-Reverse
191 (RV) 5'- TGATTCCGAAGTTGGTGGGC -3'; *Angiotensinogen* (*Agt*)-FW 5'-
192 TGCTTGGTTCACCAGGGGATA -3'; *Agt*-RV 5'- GGATGTATACGCGGTCCCCA -3';
193 *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*)-FW 5'-
194 CAACTCCCTCAAGATTGTCAGCAA -3'; *Gapdh*-RV 5'-
195 GGCATGGACTGTGGTCATGA -3'.

196 **Transthoracic Echocardiography (TTE)**

197 TTE was performed in 21-day old rats as previously described [7]. Firstly, the rats were
198 anesthetized 80 mg/kg Ketamine hydrochloride (AuroMedics Pharma LLC., Ireland) and
199 10 mg/kg Diazepam (Hospira, Inc., IL, USA). TTE was performed using the VIVID q-

200 system (GE Healthcare, Germany) equipped with a 13 MHz probe (12S-RS, GE
201 Healthcare, Germany), with the animals in left lateral decubitus, using M-mode imaging
202 of the parasternal short-axis (papillary level) view. Values were determined by averaging
203 the measurements of three consecutive cardiac cycles in accordance with the American
204 Society of Echocardiography guidelines. The above measurements were used to calculate
205 left ventricular mass (LVM) using the equation below and indexed by body weight
206 (LVMI). Additionally, the systolic functionality was calculated as the ejection fraction
207 (EF, %).

208

209 $$LVM = 0.8[1.04(IVSd + LVIDd + PWd)^3 - LVIDd^3] + 0.6$$

210 LVIDd, left ventricular end-diastolic internal diameter; LVIDs, left ventricular end-
211 systolic internal diameter; IVDs, interventricular septum thickness at diastole and PWd,
212 posterior wall thickness at diastole.

213 **Statistical Analysis**

214 Statistical analysis of body and hearth weight, LVMI and EF, were performed with IBM
215 SPSS Statistics software (version 26.0.0.0). Shapiro-Wilk test was used to test normality,
216 and analysis of the variance were performed by one way ANOVA, Turkey´s test for
217 multiple comparisons.

218 The package DESeq2 (Galaxy Version 2.11.40.2) was used to formulate RNAseq
219 differential expression of transcripts and the Benjamini-Hochberg procedure was applied
220 to calculate the adjusted p-values in the likelihood ratio test. Gene Ontology enrichment
221 analysis (GO) was performed using PANTHER (Protein ANalysis THrough Evolutionary
222 Relationships) using hypergeometric statistical test, FDR P-value correction [26,27].

223 The qPCR expression levels were analyzed with IBM SPSS Statistics software (version
224 26.0.0.0). Interaction between the factors "Litter Condition" and "Breastfeeding
225 condition" were assessed by a two-way ANOVA test. Expression levels at 21 days
226 between groups defined by the interaction of the factors "Litter Condition on
227 Breastfeeding condition" determined as "Control on Control" (Control), "MUN on
228 MUN" (MUN), "Control on MUN" (C on MUN), "MUN on Control" (MUN on C) were
229 analyzed by Kruskal–Wallis test by ranks with pairwise comparison.

230 A p-value (p) < 0.05 was considered significant.

231

232

233 **RESULTS**

234 **Fetal undernutrition induces low birth weight in both sexes, but only male rats**
235 **exhibit heart hypotrophy.**

236 The animal model of fetal undernutrition and catch-up growth has been previously
237 characterized by our laboratory. It has been demonstrated that MUN pups are born with
238 low birth weight, both in males and females. Additionally, both sexes experience
239 accelerated growth during the first 3 weeks, with no differences in body weight detected
240 at weaning, indicating the occurrence of catch-up growth process [11]. The present results
241 further illustrate that MUN rats had significantly lower body weigh at day 1 compared to
242 Control rats, both males and females (Figure 1A). Heart weigh was also significantly
243 lower in MUN males and females, compared to their sex-matched counterparts (Figure
244 1B). However, the relative heart weight to body weight ratio was only significantly lower
245 in MUN males when compared to Control, with no significant difference observed
246 between MUN and Control females (Figure 1C). These data suggest a distinct response
247 to undernutrition during the fetal period in males and females, with a more adverse
248 adaptation of cardiac tissue in males, leading to hypotrophy.

249 **Fetal undernutrition induces alterations in the gene expression profile of the heart.**

250 Given the observed growth deficiency in hearts from male MUN rats, we analyzed gene
251 expression profile of left ventricle tissue from both male MUN and Control rats at birth,
252 utilizing next generation sequencing (NGS). Through NGS, we successfully identified
253 gene expression for 11,965 genes, out of which 32 displayed significant expression level
254 changes (Benjamini-Hochberg correction) between the MUN and Control rats (Figure 2
255 A and B).

256 To further investigate whether these changes in expression levels were predominantly
257 associated with a specific biological process or molecular function, we performed Gene
258 Ontology (GO) enrichment analysis. However, the GO enrichment analysis did not reveal
259 any significant changes (Fisher´s Exact) in terms of a particular biological process,
260 molecular function, or cellular component.

261 Nonetheless, these results suggest that male MUN rats already exhibit cardiac tissue
262 alterations in gene expression regulation at birth.

263 **Lactation modulates the changes in heart *Agt* and *Pparg* gene expression caused by**
264 **fetal undernutrition.**

265 Albeit no GO category was significantly overrepresented in the genes with altered
266 expression in MUN male rats at birth, two candidate genes specifically related to blood

267 pressure regulation, cardiomegaly, and fibrosis were identified. These genes are
268 *Angiotensinogen (Agt* [29,30]) and *Peroxisome proliferator-activated receptor gamma*
269 (*Pparg* [31,32]). We sought to gain insights into the temporal progression of the expression
270 of these genes using qPCR, given the evidence of alterations in the RAS system in the
271 MUN rat model [9,10] and the presence of cardiac remodeling and fibrosis at the end of
272 lactation [9]. Regarding *Pparg*, we decided to explore it since alterations in this gene have
273 been found in the placenta of women with low-birth-weight offspring [33], and in fetal
274 perirenal adipose tissue in ewes exposed to undernutrition with a sexually dimorphic
275 pattern [34], supporting the role of this gene in fetal programming. However, to our
276 knowledge, cardiovascular alterations have not been previously identified in the context
277 of fetal programming. We explored the expression pattern of *Agt* and *Pparg* genes from
278 birth to day 21 (weaning) in both MUN and Control rats using qPCR. Additionally, to
279 investigate the potential impact of lactation on gene expression, we conducted a cross-
280 fostering experiment. In this experiment, litters born from Control rats were nursed by
281 MUN mothers (C-on-MUN), while litters born from MUN rats were nursed by Control
282 mothers (MUN-on-C), as previously described [20].
283 We initiated our analysis by examining the expression of *Agt* (Figure 3 A and B). At day
284 0, no significant difference in *Agt* expression level was observed between MUN and
285 Control hearts, neither in males nor in females. However, at day 21, MUN males
286 displayed a significantly increased *Agt* expression level compared to day 0, being also
287 larger compared to day 21 Control males. This difference was not evident in MUN versus
288 Control females, as females exhibited markedly lower levels of *Agt* expression.
289 We performed a two-way ANOVA test to investigate differences in mRNA expression
290 levels of *Agt* and *Pparg*, taking into consideration the two factors analyzed in our study
291 "Litter Condition" (Control or MUN) and "Breastfeeding condition" (Condition of the
292 mother nursing the litters, Control or MUN) in the expression of datasets for male and
293 females at day 21. In the case of *Agt* mRNA expression in males, we identified a
294 significant interaction between the factors "Litter Condition" and "Breastfeeding
295 condition" ($p=0.015$; Power= 0.72). In females, although a significant interaction was
296 observed, the power of the analysis is relatively lower ($p=0.022$; Power= 0.66). Regarding
297 *Pparg* mRNA expression, a significant interaction between the factors "Litter Condition"
298 and "Breastfeeding condition" was noted in males, albeit with a low power of the analysis
299 ($p=0.023$; Power= 0.65). No significant interaction was found in females.

300 In the cross-fostering analysis, male Control rats nursed by MUN mothers (C-on-MUN)
301 did not show a significant difference in cardiac *Agt* expression level compared to Control
302 rats nursed by Control (Control). However, male MUN rats nursed by Control mother
303 (MUN-on-C) exhibited a significant decreased *Agt* expression level when compared to
304 MUN fed on MUN mother (MUN), now matching Control *Agt* expression levels (Figure
305 3A).
306 In females, the cross-fostering analysis did not reveal any significant differences between
307 Control rats nursed by MUN mothers (C-on-MUN) and Control rats nursed by Control
308 (Control) (Figure 3B). Additionally, female MUN rats nursed by a Control mother (MUN-
309 on-C) did not show a significant difference in *Agt* expression level compared to MUN
310 nursed by MUN mothers (MUN) or compared to Control females (Figure 3B).
311 Next, we examined *Pparg* expression (Figure 3 C and D). At day 0, no significant
312 differences in *Pparg* expression level were observed between females or males, regardless
313 of their group. However, at day 21, both Control males (Figure 3 C) and Control females
314 (Figure 3 D) exhibited a marked increase in cardiac *Pparg* expression compared to day 0
315 controls. In contrast, this upregulation was not evident in MUN rats. Furthermore, 21-
316 day-old MUN males and females showed notably lower *Pparg* expression level compared
317 to 21 days old Control rats.
318 In the cross-fostering analysis, male Control rats fed on MUN mothers (C-on-MUN)
319 showed a tendency towards lower *Pparg* expression, although the difference was not
320 statistically significant when compared to Control rats fed on Control mothers (Control)
321 (Figure 3C). Similarly, Male MUN rats fed on a Control mother (MUN-on-C) also
322 displayed a similar tendency of lower but not statistically significant *Pparg* expression
323 when compared to MUN fed on MUN mothers (MUN). Comparable results were found
324 in females (Figure 3 D).
325 Taken together, these findings indicate that fetal undernutrition induces alterations in the
326 gene expression levels of *Agt* and *Pparg*, and these changes are established by the end of
327 the lactation period (at 21 days). Additionally, lactation appears to modulate gene
328 expression levels, influenced by the type of breastmilk (from Control or MUN mothers).
329 **Fetal undernutrition induces cardiac hypertrophy in 21-day-old males, but this**
330 **effect can be mitigated by lactation with Control breastmilk.**
331 We have already described heart hypertrophy and systolic dysfunction in ageing male
332 MUN rats [7]. Moreover, we have evidence, that cardiac hypertrophy is already present in
333 MUN males at weaning [9]. Consequently, we sought to investigate whether the observed

334 alterations in gene expression were correlated with left ventricular hypertrophy by
335 assessing cardiac structure through transthoracic echocardiography at the weaning stage,
336 additionally evaluating if at this early age there were already signs of dysfunction.
337 Our echocardiographic analysis revealed a significant increase in the LVMI in MUN male
338 rats compared to Control males (Figure 4 A), while no differences were observed among
339 females (Figure 4 B). We also evaluated left ventricular function through EF. Our results
340 show no significant reduction of this functional parameter, neither in MUN males (Figure
341 4C), nor in females (Figure 4D) at weaning, suggesting that left ventricular hypertrophy
342 in MUN males is an early alteration which, together with the development of hypertension
343 during adult life, may contribute to the observed cardiac dysfunction in ageing.
344 Furthermore, we aimed to uncover the possible role of breastmilk origin in heart
345 hypertrophy and whether this alteration could be mitigated through lactation, akin to the
346 observed effects on *Atg* and *Pparg* expression levels. To achieve this, we conducted
347 transthoracic echocardiography again on 21-day-old males and females in the cross-
348 fostering experiment. Remarkably, MUN males with lactation from a Control mother
349 (MUN-on-C) exhibited significantly lower LVMI compared to males lactating from
350 MUN rats. We observed no significant differences in LVMI in C-on-MUN males when
351 compared to MUN-on-C, or Controls (Figure 4 A). In females, no significant differences
352 were observed between the groups (Figure 4 B). Cross-fostering did not modify
353 significantly EF in any of the groups (Figure 4C, 4D).
354 These findings indicate that the cardiac alterations observed in MUN rats, are sex-
355 dependent, and can be modulated by lactation environment, exhibiting a parallelism with
356 *Agt* expression.

357 **DISCUSSION**

358 Using the well-characterized MUN rat animal model, which develops cardiac alterations
359 associated with fetal growth restriction [7,9], we have identified specific gene expression
360 pattern alterations in the heart caused by fetal programming**.** Our study further
361 investigated *Agt* and *Pparg*, genes related to cardiac hypertrophy, and found that their
362 expression is modulated during lactation period. The results from our cross-fostering
363 experiments demonstrated that changing breastmilk origin can diminish the impact of
364 fetal programming on gene expression and heart hypertrophy. These findings suggest that
365 lactation period could serve as an intervention window to mitigate or revert adverse
366 consequences initiated during the intrauterine period programmingcardiometabolic
367 diseases.

368 **Heart remodeling involves the alteration of several groups of genes in fetal**
369 **programming.**
370 Our study revealed that exposure to undernutrition during intrauterine period modifies
371 several heart genes expression, however we did not find specific modifications in a
372 particular group of genes related to a specific biological process. Numerous studies have
373 identified heterogeneous genes in specific tissues that alter their expression in response
374 to fetal undernutrition. For instance, maternal undernutrition during gestation in cattle has
375 been shown to alter gene expression associated with energy metabolism and angiogenesis,
376 specifically in skeletal muscle [35]. Studies in ovine animal models of fetal programming
377 showed gene expression alteration of cell cycle and apoptosis in liver [36], while studies in
378 rats have shown IGF-1 gene expression alterations in liver [37]. These findings suggest that
379 fetal programming caused by undernutrition, rather than affecting specific processes or
380 pathways, could alter gene regulatory region accessibility, disturbing several genes which
381 together contribute to a phenotype.
382 Among the genes modified by fetal undernutrition, two candidates emerged as promising
383 targets for exploration, given the alterations we have previously found in heart for MUN
384 males [7], which were already evident at weaning [9,20]. At day 21 in the present study, the
385 end of the lactation period, we observed an increase of *Agt* expression in MUN male rats.
386 Angiotensinogen is the initial substrate of RAS, ultimately leading to Ang II, a well-
387 known stimulus for cardiac hypertrophy [38]. In contrast, we also observed lower levels of
388 *Pparg* expression in the same MUN male rats. PPARs, including PPARγ , are relevant
389 transcription factors in the regulation of cardiac energy metabolism and mitochondrial
390 function, and their stimulation has been proposed for the management of cardiometabolic
391 diseases [32]. Specifically, *Pparg* plays a crucial role in cardiac tissue development by
392 increasing its expression after birth, inducing the expression of genes encoding Fatty Acid
393 Oxidation (FAO) enzymes and other proteins related to mitochondrial energy production
394 pathways [39]. Thus in the heart, PPARs serve as physiological master switches that
395 modulate cardiac energy metabolism, being particularly relevant in the transition from
396 intrauterine to extrauterine life. In the initial days post-birth, the increase in oxygen partial
397 pressure and the delivery of fatty acids from breast milk shift mitochondrial lipid
398 oxidation to become the primary source of ATP. PPARs play a pivotal role in orchestrating
399 this metabolic switch as evidenced by their substantial rise after birth [32]. This
400 physiologically elevated expression of *Pparg* gene was observed in the hearts of control
401 rats from postnatal day 1 to day 21, signifying its importance in the cardiac metabolic

402 adaptation. However, this elevation was notably absent in MUN rats. Beyond its cardiac
403 metabolic role, PPARγ has been implicated as a growth suppressor, as demonstrated by
404 cardiac hypertrophy in mice with cardiomyocyte-specific PPARγ deficiency [40]. These data
405 support a role of *Pprg* gene expression alterations in the observed heart hypertrophy in
406 MUN male, also associated with oxidative damage at weaning in MUN model [41].
407 Nevertheless, the role of PPRs in fetal programming is complex, as both deficient
408 upregulation after birth and *Pparg* overexpression during the intrauterine period have
409 been associated with fetal cardiac abnormalities. Alterations in *Pparg* signaling pathway
410 may account for these discrepancies. Interestingly, there is a deactivation of the
411 PPARG/PGC-1 complex during pathological hypertrophic growth, accompanied by a
412 decrease of the expression of *Pparg* and the response of its responsive element NRRE-1
413 activity [42]. Reduced PPARγ signaling is also associated with transforming growth factor
414 beta (TGFβ), implicated in fibrosis and cardiovascular remodeling [43]. Since we have
415 evidence of increased fibrosis and TGFβ in male MUN hearts at the age of 21 days [9], we
416 suggest that the reduction of *Pparg* gene expression may also contribute to the observed
417 cardiac hypertrophy. Additionally, in hearts from adult mice exposed to fetal
418 overnutrition, which also induces cardiac hypertrophy, GO analysis evidenced a
419 downregulation of *PPRa* (a related factor) and upregulation of TGFβ signaling-related
420 genes [44]. Thus, our results in MUN males evidencing increased LVMI at the end of
421 lactation, are in line with previous data on the role of these genes, and support that an
422 increase of *Agt* and a decrease of *Pparg* expression levels during perinatal life contribute
423 to cardiac hypertrophy. Likewise, even though MUN females showed a similar decrease
424 in *Pparg,* they did not show the increase in *Agt* expression and did not develop heart
425 hypertrophy, suggesting the role of both genes in the development of the final phenotype.
426 Besides, while females exhibit no signs of cardiac hypertrophy, we cannot discard the
427 possibility of early fibrosis, which in the long run may contribute to the later development
428 of cardiac hypertrophy in aged MUN females [7].
429 In addition to *Pparg* and *Agt* , RNAseq analysis also evidenced alterations in the
430 expression of other genes which may potentially contribute to cardiac remodeling
431 process. For example, *Gdf6* (growth differentiation factor 6) was downregulated in our
432 study at day 0. *Gdf6* is a mediator of vascular remodeling induced by Ang II through
433 adventitial fibroblast *Nox2*, a relevant ROS-producing enzyme [45]. Oxidative stress and
434 elevated Ang II are evidenced at early age in MUN male heart [41], and therefore, it would

435 be interesting to explore the participation of this gene in the development of cardiac
436 hypertrophy in this rat model. Fat-1 was upregulated in MUN males at birth. This gene
437 codifies for the enzyme converting omega-6 (n-6) to omega-3 (n-3) polyunsaturated fatty
438 acid and there is evidence that the offspring of obese dams expressing the *Fat-1* transgene
439 show a lower post-weaning weight gain [46]. We have evidence of catch-up growth and fat
440 accumulation in MUN offspring during lactation [47]; thus, it remains to be elucidated if
441 *Fat-1* gene expression is modified in undernourished rats during postnatal period and the
442 impact of lactation environment. Some of the altered genes differentially expressed at
443 birth are associated with DNA repair. Among them, Agpat3, which has been involved in
444 pathways encoding for energy, phagocytosis, and DNA repair in response to cigarette
445 smoke [48] and APE2, an important endonuclease also implicated in DNA damage-repair
446 processes [49] . We cannot discard that altered regulation of these genes may participate in
447 the cardiac remodelling preocess in MUN rats, since there is evidence ofcardiac DNA
448 damage, oxidative and nitrosative stress [50] in a rat model of maternal undernutrition
449 induced by low protein diet during gestation, with similar features to MUN. Tarry-Adkins
450 and co-workers found that fetal undernutrition followed by catch-up growth increased
451 cardiac expression of some genes related to DNA damage-repair processes at birth,
452 although they were not effective with the result of oxidative and nitrosative damage at
453 weaning. Their results have strong similarities to those previously reported in MUN males
454 [41] . Therefore, it would be interesting to explore the evolution in the expression of these
455 genes during lactation.

456 **Lactation period is a key factor in stablishing gene expression modification caused**
457 **by fetal programming.**

458 Our study revealed that MUN offspring exhibited changes in gene expression at birth,
459 likely due to epigenetic modulation, which aligns with previous studies in fetal
460 programming [13–15]. However, one of the most significant findings from our study,
461 revealed by the Cross-fostering analysis, is the important role that lactation period plays
462 in modifying gene expression in the MUN model. The differences in *Agt* and *Pparg*
463 expression levels observed between Control and MUN male offspring (lactating from
464 their respective mothers) completely disappeared in Cross-fostering experiment. This
465 evidence indicates that *Agt* expression of MUN male offspring with access to breastmilk
466 from Control dams was reduced to levels comparable to controls. Furthermore, MUN
467 male offspring with access to Control breastmilk did not develop heart hypertrophy. These
468 results highlight, form the first time, a synergy between fetal programming and lactation,

469 and how both contribute to the development of cardiometabolic diseases, through the
470 modulation of gene expression. Previous studies have demonstrated that epigenetic
471 changes could be transmitted through breastmilk components [51]. Additionally, it is
472 known that folic acid supplementation to rodent mothers could directly modify offspring
473 gene expression [52]. Moreover, Control offspring without prior programming did not
474 modify *Agt* expression or cardiac LVMI by changing the lactation environment. Thus,
475 together these results in a model of fetal programming of cardiac hypertrophy and
476 hypertension support the notion that gene expression changes at birth are regulated by
477 factors present in breastmilk during lactation period, and both fetal and perinatal life
478 influences are necessary to stablish the final phenotype.

479 **Lactation as an intervention window to prevent or counter fetal programming.**

480 The ability to reverse heart hypertrophy in MUN offspring simply by giving them access
481 to Control breastmilk in cross-fostering experiments opens the possibility of using
482 lactation period as intervention window to counteract fetal programming, as recently
483 proposed [14]. However, the specific factors present in breast milk and the mechanisms
484 causing the epigenetic changes remain unknown. It is important to highlight that in bovine
485 model, the digestion of caseins present in breastmilk generates casein-derived peptides
486 that can inhibit angiotensin-converting enzyme [53], which could potentially contribute to
487 mitigate heart hypertrophy, but does not explain the gene expression changes observed in
488 our MUN model. On the other hand**,** breastmilk is known to contain a multitude of active
489 compounds, such as exosomes, which can have a significant impact on several processes
490 as immunity, growth and development, cell proliferation, apoptosis or differentiation of
491 progenitor cells [54]. Additionally, the presence of microRNAs in breastmilk has been
492 described to directly modify the expression of several genes, including *INS*, *IGF1*, *NRF2*,
493 *GLUT1* and *FOXP3* genes [55]. Breastmilk also contains hormones and essential fatty acids,
494 which are modulated by maternal nutritional status, as we have previously reported in
495 humans [56,57], and recent evidence highlights that these compounds are relevant
496 modulators of gene expression and cardiac growth. For example, circulating aldosterone
497 causes DNA demethylation of the promoter region of *Agt,* increasing its expression in the
498 heart [58]. A recent study emphasizes the modulatory role of breastmilk γ-linolenic acid as
499 a key transcriptional regulatory mechanism of perinatal cardiac growth and metabolism
500 [59]. Future studies should focus on identifying specific components present or absent in
501 MUN breast milk compared to Control which could correlate with the genetic changes
502 we observed.

503 Currently, epigenetic modifications contributing to the development of cardiometabolic
504 diseases can only be interfered by amendments in risk factors as stress, sleep deprivation,
505 obesity and sedentarism [14]. Uncovering the components or pathways that are susceptible
506 to intervention through lactation would open a new way of treatment potentially more
507 effective in addressing fetal programming.

508 **Limitations of the study.**

509 The present study aimed to explore alterations in the cardiac gene expression patterns
510 induced by undernutrition during fetal life, potentially linked to previously reported
511 ventricular hypertrophy in male offspring at weaning. Our secondary objective was to
512 analyze the influence of the lactation environment on the modulation of relevant genes.
513 Several genes emerged in the RNAseq analysis, from which we chose to focus on *Agt* and
514 *Pprg*. While we found evidence of dysregulation in these genes during lactation period,
515 coinciding with cardiac hypertrophy, it is crucial to acknowledge the potential
516 involvement of other genes identified in RNAseq analysis, such as *Agpat3*, *GDF6* and
517 *Fat-1*. This limitation warrants further exploration in subsequent studies.

518

519 A second limitation lies in the exclusive evaluation of gene expression levels, without
520 concurrent assessment of protein expression. Additionally, attention should be directed
521 towards changes in breastmilk components between MUN and control dams. Although
522 we observed a lower, albeit statistically insignificant, fat content in MUN milk in our
523 previous study, a detailed examination of its composition in various fatty acids is
524 essential. This becomes particularly pertinent in light of the observed deficient *Pprg*
525 expression in MUN rats, raising questions about a potential inadequate switch to fatty
526 acid oxidation during lactation.

527 **DATA AVAILABILITY**

528 The datasets generated during and/or analysed during the current study are available from
529 the corresponding author on reasonable request.

530

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AUTHOR CONTRIBUTIONS

I.M.C.-R.G.-P.R-A.S-S.M.A.: data acquisition and data analysis (animal model, tissue dissection). P.R-B.Q.U.: data acquisition, analysis, and interpretation (echocardiography). I.M.C.-R.G.-J.M.G.-S.M.A.: data acquisition, analysis, and interpretation (RNAseq analysis). I.M.C.-R.G.-J.M.G.-S.M.A.: data acquisition, analysis, and interpretation (qPCR analysis) I.M.C.-J.M.G.-S.M.A.: conception or design of the work, drafting of the manuscript and project administration. All authors have read and agreed to the published version of the manuscript.

COMPETING INTEREST

There are no potential conflicts of interest.

INSTITUTIONAL BOARD REVIEW STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and it was approved by the Ethics Review Boards of Universidad Autónoma de Madrid (CEI-UAM 96-1776-A286) and the Regional Environment Committee of the Comunidad Autónoma de Madrid (RD 53/2013; Ref. PROEX 04/19; date 19 March 2019).

FIGURES

Figure 1. Body and heart weighs at birth in MUN male and female rats. Data are expressed as mean ± SEM, n = 11 rats per group. The figure represents body weight A), heart weight B) and relative heart weight C) at 1d. Statistical analysis was performed by one way ANOVA Turkey´s test for multiple comparisons; *p < 0.05 **p<0.01 compared to sex-matched Control; C= Control, MUN= Maternal Undernutrition.

Figure 2. Differential gene expression at birth in MUN male rats. A) Volcano Plot from the RNAseq gene expression data. Significant hits are depicted in red (T-test Benjamini-Hochberg correction). B) Table representing upregulated and downregulated gene expression in MUN male rats at birth when compared to Control.

704 **Figure 3. Differential *Agt* and *Pparg* expression in MUN and Cross-fostered rats.**
705 The data show mRNA comparative expression in Control (C) and maternal undernutrition
706 (MUN) litters, at day 0 and day 21 and in a cross-fostering experiment condition, where
707 litters born from Control rats suck milk from MUN mothers (C on MUN) and litters born
708 from MUN rats suck milk from Control mothers (MUN on C) at day 21. The data
709 represents *Agt* expression in males A) and females; and *Pparg* expression in males C) and
710 females D). * $p < 0.05$ compared to sex-matched 0 day Control rats. #$p<0.05$ compared
711 to sex-matched 21day Control rats.
712 **Figure 4. Left Ventricular Mass Index (LVMI) and Ejection Fraction (EF) in MUN**
713 **and Cross-fostered rats.**
714 Left ventricular mass index (LVMI) calculated as left ventricular mass/body weight (A
715 and B) and ejection fraction (EF) in Control (C) litters, maternal undernutrition (MUN)
716 litters and cross-fostering experiment condition, where litters born from Control rats suck
717 milk from MUN mothers (C on MUN) and litters born from MUN rats suck milk from
718 Control mothers (MUN on C) at day 21 of males A and C) and females B and D).
719 **$p<0.01$ compared to sex-matched Control rats.
720

721 **Supplementary Table 1. Body and heart weight at birth.**

	n	**Body mean weight (g)**	**Body S.D.**	**Heart Mean weight (g)**	**Heart S.D.**
C males	11	5.094	0.648	0.041	0.0085
MUN Males	11	3.204	0.723	0.022	0.0066
C Females	12	3.783	0.209	0.021	0.0037
MUN Females	12	2.743	0.456	0.015	0.0041

722

723 **Supplementary Table 2. *Agt* and *Pparg* mRNA expression.**

	n (*Agt*)	***Agt* mRNA**	***Agt* mRNA S.D.**	**n (*Pparg*)**	***Pparg* mRNA**	***Pparg* mRNA S.D.**
C males d0	3	0.7545	0.424	3	0.040	0.024
MUN males d0	3	0.875	0.410	3	0.062	0.065
C males d21	8	1.115	0.617	7	1.153	0.869
MUN males d21	7	2.068	0.889	8	0.510	0.360
C on MUN males d21	3	0.662	0.701	3	0.165	0.257
MUN on C males d21	3	0.627	0.543	3	0.071	0.049
C females d0	3	0.400	0.213	3	0.083	0.057

MUN females d0	3	0.859	0.259	3	0.172	0.036
C females d21	7	0.818	0.320	9	0.909	0.754
MUN females d21	8	0.927	0.454	9	0.150	0.009
C on MUN females d21	3	0.217	0.037	3	0.046	0.018
MUN on C females d21	3	0.605	0.431	3	0.175	0.207

724

725 **Supplementary Table 3. LMVI (left ventricular mass index) and EF (ejection**
726 **fraction) at 21 days.**

	N (LMVI)	LMVI (mg/g)	LMVI S.D.	n (EF)	EF %	EF S.D.
C males	5	12.68	1.204	5	94.40	2.302
MUN males	4	16.34	1.485	5	94.20	4.868
C on MUN males	6	11.62	0.920	5	90.89	5.541
MUN on C males	6	14.10	2.353	5	95.67	2.727
C females	6	12.75	1.463	6	93.83	1.472
MUN females	3	11.69	0.475	4	93.00	0.816
C on MUN females	6	11.21	0.885	6	91.01	4.628
MUN on C females	5	13.38	1.705	6	90.58	7.451

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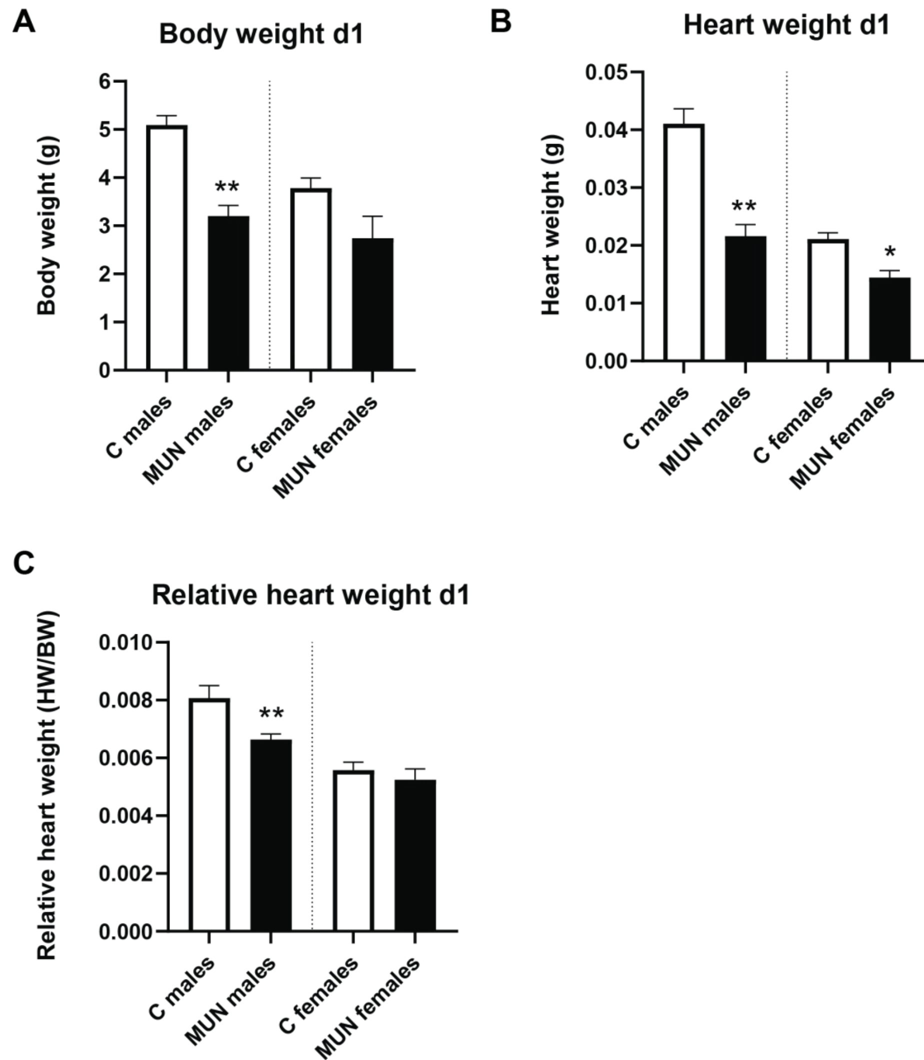

Figure 1. Body and heart weighs at birth in MUN male and female rats. Data are expressed as mean ± SEM, n = 11 rats per group. The figure represents body weight A), heart weight B) and relative heart weight C) at 1d. Statistical analysis was performed by one way ANOVA Turkey´s test for multiple comparisons; *p < 0.05 **p<0.01 compared to sex-matched Control; C= Control, MUN= Maternal Undernutrition.

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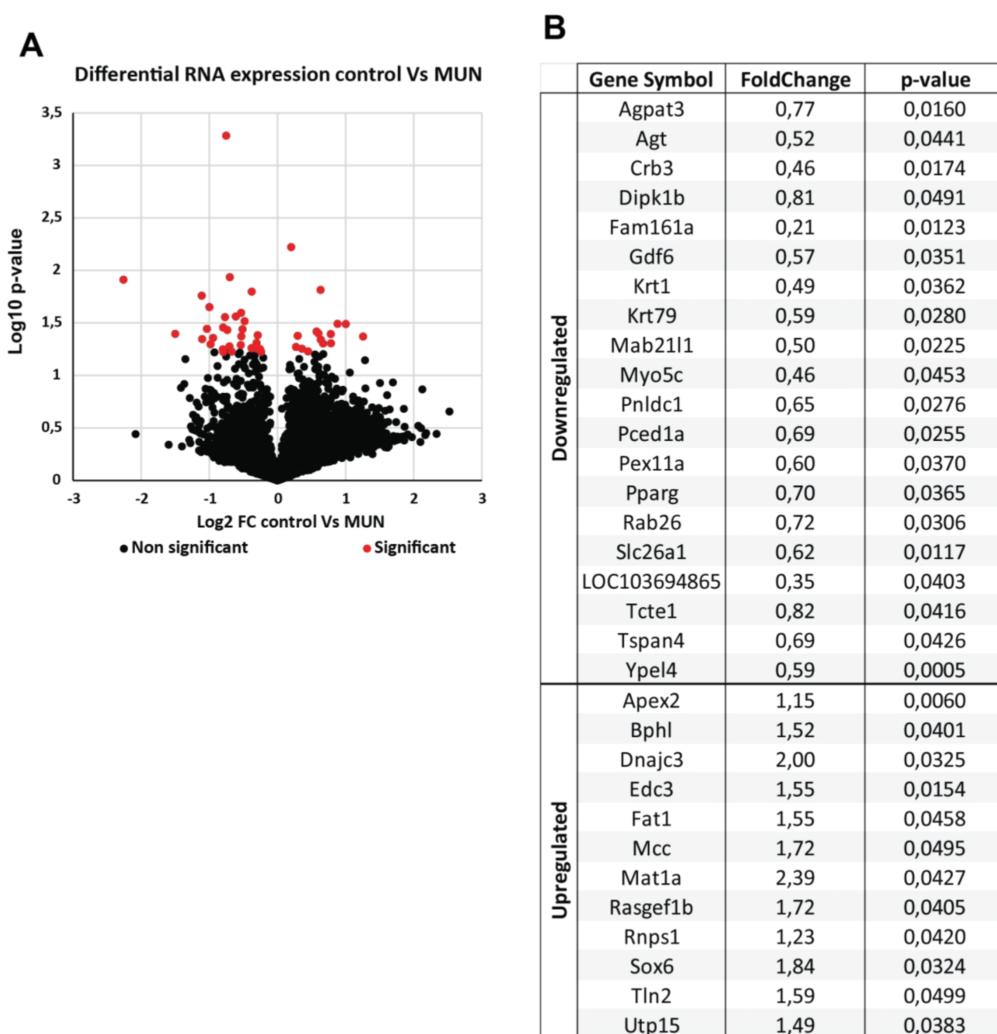
FIGURE 2.



	Gene Symbol	FoldChange	p-value
Downregulated	Agpat3	0,77	0,0160
	Agt	0,52	0,0441
	Crb3	0,46	0,0174
	Dipk1b	0,81	0,0491
	Fam161a	0,21	0,0123
	Gdf6	0,57	0,0351
	Krt1	0,49	0,0362
	Krt79	0,59	0,0280
	Mab21l1	0,50	0,0225
	Myo5c	0,46	0,0453
	Pnldc1	0,65	0,0276
	Pced1a	0,69	0,0255
	Pex11a	0,60	0,0370
	Pparg	0,70	0,0365
	Rab26	0,72	0,0306
	Slc26a1	0,62	0,0117
	LOC103694865	0,35	0,0403
	Tcte1	0,82	0,0416
	Tspan4	0,69	0,0426
	Ypel4	0,59	0,0005
Upregulated	Apex2	1,15	0,0060
	Bphl	1,52	0,0401
	Dnajc3	2,00	0,0325
	Edc3	1,55	0,0154
	Fat1	1,55	0,0458
	Mcc	1,72	0,0495
	Mat1a	2,39	0,0427
	Rasgef1b	1,72	0,0405
	Rnps1	1,23	0,0420
	Sox6	1,84	0,0324
	Tln2	1,59	0,0499
	Utp15	1,49	0,0383

Figure 2. Differential gene expression at birth in MUN male rats. A) Volcano Plot from the RNAseq gene expression data. Significant hits are depicted in red (T-test Benjamini-Hochberg correction). B) Table representing upregulated and downregulated gene expression in MUN male rats at birth when compared to Control.

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FIGURE 3.

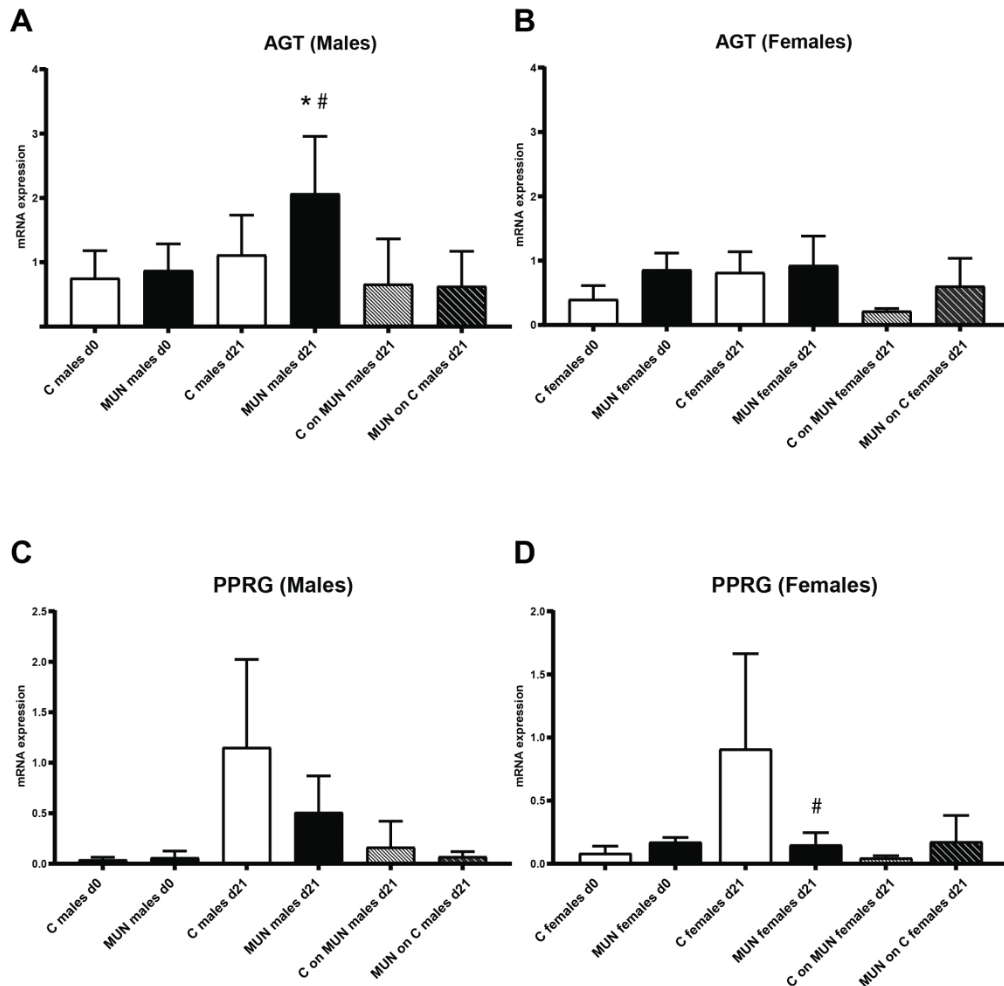

Figure 3. Differential Agt and Pparg expression in MUN and Cross-fostered rats.
The data show mRNA comparative expression in Control (C) and maternal undernutrition (MUN) litters, at day 0 and day 21 and in a cross-fostering experiment condition, where litters born from Control rats suck milk from MUN mothers (C on MUN) and litters born from MUN rats suck milk from Control mothers (MUN on C) at day 21. The data represents Agt expression in males A) and females; and Pparg expression in males C) and females D). * $p < 0.05$ One-way ANOVA Tukey's multiple comparisons test.

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FIGURE 4.

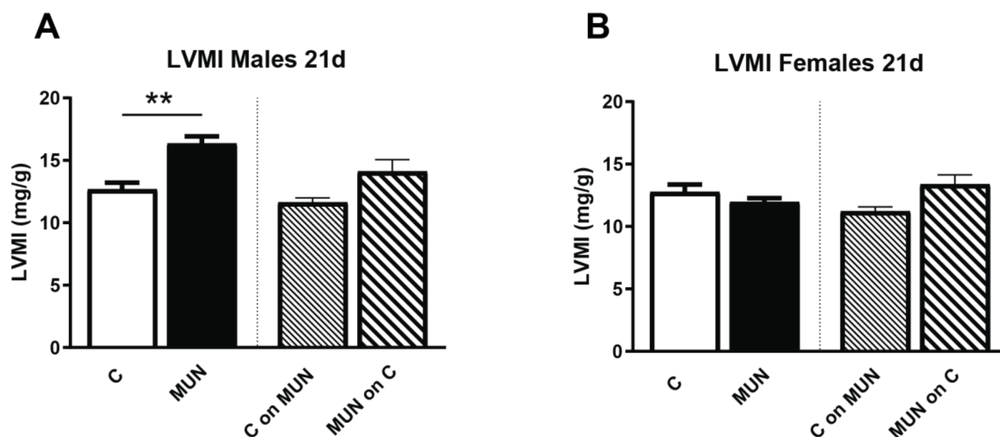

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Figure 4. Left Ventricular Mass Index (LVMI) in MUN and Cross-fostered rats.
Left ventricular mass index (LVMI) calculated as left ventricular mass/body surface in Control (C) litters, maternal undernutrition (MUN) litters and cross-fostering experiment condition, where litters born from Control rats suck milk from MUN mothers (C on MUN) and litters born from MUN rats suck milk from Control mothers (MUN on C) at day 21 of males A) and females B). *$p<0.05$ compared to sex-matched 0 day Control rats. #$p<0.05$ compared to sex-matched 21day Control rats.

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