



HAL
open science

Early life nutrition influences thymic growth in male mice that may be related to the regulation of longevity

Jian-Hua Chen, Jane L. Tarry-Adkins, Chantal A. A. Heppolette, Donald B. Palmer, Susan E. Ozanne

► **To cite this version:**

Jian-Hua Chen, Jane L. Tarry-Adkins, Chantal A. A. Heppolette, Donald B. Palmer, Susan E. Ozanne. Early life nutrition influences thymic growth in male mice that may be related to the regulation of longevity. *Clinical Science*, 2009, 118 (6), pp.429-438. 10.1042/CS20090429 . hal-00546866

HAL Id: hal-00546866

<https://hal.science/hal-00546866>

Submitted on 15 Dec 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Early life nutrition influences thymic growth in male mice that may be related to the**
2 **regulation of longevity**

3
4 Jian-Hua Chen^{1*}, Jane L. Tarry-Adkins¹, Chantal A. A. Heppolette¹, Donald B. Palmer²
5 and Susan E. Ozanne¹
6
7

8 **1** University of Cambridge Metabolic Research Laboratories, Institute of Metabolic
9 Science, Cambridge, CB2 0QQ, U.K. **2** Royal Veterinary College, Infection & Immunity
10 and Genes & Development Group, Department of Veterinary Basic Sciences, London,
11 NW1 0TU, U.K.
12
13

14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31 **Keywords:** Maternal diet, Thymic growth, Thymic involution, Immunosenescence,
32 Lifespan, Developmental programming.
33

34 **Short title:** Early life nutrition influences thymic growth
35
36

37 * Author for correspondence: Jian-Hua Chen (jhc36@cam.ac.uk)

38

39

Abstract

40 Nutrition and growth rate during early life can influence later health and lifespan. We
41 previously demonstrated that low birth weight resulting from maternal protein restriction
42 during pregnancy followed by catch-up growth in rodents was associated with shortened
43 lifespan whereas protein restriction and slow growth during lactation increased lifespan.
44 The underlying mechanisms by which these differences arise are unknown. Here we
45 report that maternal protein restriction in mice influences thymic growth in early adult
46 life. Offspring of dams fed a low protein diet during lactation (PLP offspring) showed
47 significant thymic growth from 21 days to 12 weeks of age whereas this was not observed
48 in control mice or offspring of dams fed a low protein diet during pregnancy (recuperated
49 offspring). PCNA and SIRT1 protein levels at 21 days of age were significantly higher in
50 thymus from both PLP ($P < 0.001$ and $P < 0.05$ respectively) and recuperated mice ($P <$
51 0.001 and $P < 0.01$ respectively) compared to controls. At 12 weeks PLP maintained a
52 higher SIRT1 level whereas PCNA was decreased in thymus from recuperated offspring.
53 This suggests that mitotic activity was initially enhanced in thymus of both PLP and
54 recuperated offspring but remained sustained into adulthood only in PLP mice. The
55 differential mitotic activity in thymus of PLP and recuperated animals appeared to be
56 influenced by changes in sex hormone concentrations and expression of p53, p16, the
57 androgen receptor, IL-7 and the IL-7 receptor. The differential thymic growth may
58 contribute to the regulation of longevity by maternal diet.

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090429

Accepted Manuscript

59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104

Introduction

It is well known that early growth patterns can have profound long-term effects on later health. Epidemiological studies first demonstrated a close link between low birth weight and later adult cardiovascular disease [1]. Subsequent studies further established that poor fetal growth is associated with later risk of adult diseases including cardiovascular disease, metabolic syndrome and type 2 diabetes [2]. This led to the concept of the fetal/early origins of adult disease which is now widely known as developmental origins of health and disease [3]. The developmental origins hypothesis proposes that during critical periods of fetal and postnatal mammalian development, nutrition and other environmental factors induce adaptive responses. These lead to permanent changes in metabolism, hormone production and tissue sensitivity to hormones that may affect development of various organs, resulting in long-term alteration of physiology and metabolism and ultimately adult disease susceptibility [4].

Animal models that allow manipulation of fetal and postnatal growth rates not only supported the developmental origins hypothesis but also provided insight into the underlying mechanisms by which early growth affects later adult health [4,5]. Using rodent models of maternal protein restriction we showed that a poor early nutritional environment can lead to the development of insulin resistance and diabetes in later adult life [6]. We also demonstrated that maternal protein restriction can ultimately affect longevity. Low birth weight resulting from maternal protein restriction during pregnancy followed by postnatal catch-up growth (recuperated animals) was associated with shortened lifespan [7,8]. Conversely, protein restriction and slow growth during lactation (postnatal low protein animals: PLP) increased lifespan [7,8].

The immune system is essential to defend the body against attacks by foreign organisms and pathogens, and thus is vital for health and longevity. The development of the immune system involves a complex series of events that occurs during fetal and early postnatal periods of mammalian growth. As fetal and early postnatal life is a critical time for development of a functional immune system [9], it may be sensitive to maternal diet during pregnancy and lactation [10]. Indeed, human studies have suggested that the early nutritional environment and growth patterns can have an impact on long-term immune function [11,12].

The thymus is the principal organ which is responsible for the generation of circulating T lymphocytes and hence plays an important role in cellular immunity. It is well known that the thymus regresses with age (age-associated thymic involution), which is characterized by a reduction in thymic mass, loss of tissue structure, abnormal architecture, and a decline in thymocyte numbers leading to a reduction in naïve T cell output [13-15]. It is this decline in the number of T cells exiting the thymus that is believed to significantly contribute to the reduced response to vaccination and infections and increased incidence of cancers and autoimmune diseases in advancing age [16-18]. Thus thymic involution can be regarded as one of the leading regulators of aging [19]. Indeed, caloric restriction which extends lifespan in species ranging from worms and flies to rodents can retard immune senescence; possible by delaying thymic involution [20]. Moreover, a recent study clearly demonstrated that genetically modified long-lived mice were resistant to age-dependent thymic involution and maintained a better immune homeostasis during postnatal development and aging [21].

105 We hypothesize that development of the thymus may be influenced by maternal
106 diet and early growth patterns, which could potentially contribute to the regulation of the
107 aging process and ultimately affect lifespan. The aim of this study was therefore to
108 investigate the effects of maternal diet on thymic mass in mice and to explore potential
109 underlying mechanisms. In this study we subjected mice to exactly the same maternal
110 protein restriction regime that is known to influence lifespan [8]. Here we report
111 differential growth of the thymus and changes in sex steroid hormone concentrations and
112 expression of genes that are involved in thymic growth and involution during early adult
113 life in our maternal protein restriction mouse models.

Accepted Manuscript

114

Materials and Methods

115

Animals

117 All animal procedures were carried out in compliance with the British Home Office
118 Animals Act (1986). Mice (C57/b16) were bred locally at a designated animal unit of the
119 University of Cambridge (Cambridge, UK). Adult female mice were housed individually
120 and were maintained at 22°C on a 12:12 hours light:dark cycle. They were mated at six
121 weeks of age (~ 22 g in body weight) and assumed to be pregnant when a vaginal plug
122 was expelled. Dams were fed *ad libitum* either a control diet (containing 20% protein) or
123 an isocaloric low protein diet (containing 8% protein) during gestation and lactation. Both
124 the control and low protein diet were purchased from Arie Blok (Woerden, The
125 Netherlands) and detailed diet composition was provided earlier [22]. Cross-fostering
126 techniques were used two days after birth to establish three groups: control (offspring
127 born to and suckled by control diet fed dams), postnatal low protein (PLP: offspring of
128 control diet fed dams nursed by low protein diet fed dams), and recuperated (offspring of
129 dams fed the low protein diet and nursed by control diet fed dams). A low protein diet
130 during pregnancy had no statistically significant effect on litter size: pups per litter: low
131 protein, 6.7 ± 0.4 ; control, 7.4 ± 0.4 . To maximize the effects of maternal diet differences
132 on offspring growth, recuperated pups were culled to 4 (to maximize growth during
133 suckling) and control pups were culled to 8 (if the number of pups was greater than 8)
134 whereas PLP pups were uncultured (to reduce the plane of nutrition). Litter size
135 standardization was carried out randomly. Pups were weaned on to a standard laboratory
136 chow diet at 21 days of age. Animals were starved overnight before tissue collection. One
137 male was selected at random from each litter at each age (21 days and 12 weeks). Fasting
138 blood was collected following decapitation, allowed to clot for 30 min, and then
139 centrifuged at 1,000 g for 6 min to obtain serum. Thymic weights at 21 days and 12
140 weeks of age were monitored by weighing the tissues from overnight fasted male mice.
141 Tissues were snap frozen in liquid nitrogen and stored at -80°C until analysis.

142

Measurements of testosterone and estradiol

144 Due to limited serum volume from individual animals and serum volume requirements
145 for the assays the only feasible way to measure testosterone and estradiol was using
146 pooled samples. We pooled the serum samples with equal aliquots (35 μ l) from nine mice
147 in each group. Mouse serum samples were extracted with ten times volume of ether.
148 Following ether being fully evaporated, the extracted testosterone was reconstituted in
149 assay buffer and assayed in duplicate using Coat-A-Count Total Testosterone RIA kits
150 according to manufacturer's protocols (Diagnostic Products Corporation, Los Angeles,
151 CA, USA; Coat-a-Count Kit Total Testosterone Manual, #PITKTT-4, 2005-03-18).
152 Estradiol in mouse serum samples were assayed in duplicate using an AutoDELFLIA
153 Estradiol kit (Perkin Elmer Life Sciences, Wallac Oy, Turku, Finland) according to
154 manufacturer's protocols.

155

Western blotting

157 Total protein was extracted from mouse thymus by homogenization in ice-cold lysis
158 buffer [50 mM HEPES (pH 8), 150 mM NaCl, 1% Triton X100, 1 mM sodium
159 orthovanadate, 30 mM NaF, 10 mM sodium pyrophosphate, 10 mM EDTA, and a

160 protease inhibitor cocktail set III (Calbiochem Novabiochem Biosciences, Nottingham,
161 UK)]. The total protein concentration in the lysates was determined using a Sigma copper
162 sulfate/bicinchoninic acid assay (Sigma, Dorset, UK). Samples were diluted to a
163 concentration of 1 mg/ml in Laemmli's buffer. Twenty micrograms of total protein were
164 subjected to SDS-PAGE and transferred onto PVDF immobilon-P membrane (Millipore,
165 Billerica, MA, USA). Membranes were blocked in Tris-buffered saline with 0.1%
166 Tween-20 and 5% dry milk, and incubated with the primary antibodies against:
167 proliferating cell nuclear antigen (PCNA) (ab29-100, Abcam, UK), Sirt1 (#07-131,
168 Millipore, Billerica, MA, USA) and p53 (MAB1746, R&D Systems, Minneapolis, MN,
169 USA). The bound primary antibodies were detected by horseradish peroxidase-
170 conjugated secondary antibodies (Dako, Glostrup, Denmark), followed by enhanced
171 chemiluminescence (GE Healthcare, Buckinghamshire, UK). The densities of the bands
172 were quantified using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA,
173 USA). Twenty four samples were run on a single gel alongside molecular weight markers
174 and 10 and 20 μ g of one sample were loaded onto each gel to ensure the linearity of the
175 signal. Equal loading was confirmed by Coomassie blue staining.

176 177 **Extraction of total RNA and cDNA synthesis**

178 Total RNA samples were prepared using TRI reagent (Sigma, Dorset, UK), purified using
179 RNeasy Mini Kits (Qiagen, West Sussex, UK) with a DNase digestion step included to
180 eliminate potential contaminating DNA and quantified spectrophotometrically on a
181 NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). First strand cDNA was
182 reverse transcribed from 1 μ g of total RNA using an ImProm-II Reverse Transcription
183 System (Promega, Hampshire, UK) with Oligo(dT)₁₅ as the primer according to the
184 manufacturer's protocol.

185 186 **Gene expression analysis by real-time PCR**

187 Real time quantitative PCR was carried out using an ABI PRISM 7900 Sequence
188 Detection System (Applied Biosystems, Foster City, CA, USA) with a SYBR Green PCR
189 Master Mix (Applied Biosystems, Foster City, CA, USA) and gene-specific primers.
190 Primers were custom designed and synthesized by Sigma (Sigma, Dorset, UK) and are
191 summarized in Table 1. 3 μ l of cDNA template (diluted according to relative expression
192 level of each gene of interest) were used in a 12 μ l of total reaction volume in each well
193 in a 96-well reaction plate. For every gene analyzed in this study we performed
194 dissociation curve analysis to ensure that the primers used did not form primer-dimers.
195 The transcripts of each gene were amplified in duplicate. Standard curves were
196 constructed using serial diluted pooled cDNA samples from eight control animals. The
197 relative expression levels were calculated against each gene's standard curve with the Ct
198 values of each gene for each animal with the housekeeping gene β -actin as a loading
199 control. Expression of β -actin showed equal levels between control, PLP and recuperated
200 animals.

201 202 **Statistical analysis**

203 Data were analyzed by ANOVA with maternal diet and age as the independent variables.
204 Duncan's post hoc testing was used to analyze significant differences between groups
205 where appropriate. A $P < 0.05$ was considered statistically significant.

206

207

Results

Thymic weights in young adult mice

At 21 days of age thymic weight of PLP mice was similar to that of controls (Figure 1A). In contrast, thymic weight of 21 day old recuperated mice was heavier than that of controls ($P = 0.026$, Figure 1A). At 12 weeks of age, thymic weights of both control and recuperated showed no change in weight compared to 21 days of age (Figure 1A) whereas thymic weight of 12 week old PLP mice showed a significant increase (Figure 1A; $P < 0.001$). As reported previously, PLP mice showed slow growth during lactation and had significantly lower body weights at weaning (21 days old) whereas recuperated mice although born small, underwent a catch-up growth thus at weaning their body weight was significantly higher than controls [22] (Figure 1B). Thus relative thymus weight (adjusted by body weight) in PLP mice was significantly higher than that of controls whereas the relative thymus weight in recuperated mice showed no difference compared to controls (Figure 1C). Furthermore, as the body weight of PLP mice remained lighter and recuperated mice remained heavier than controls at 12 weeks of age (Figure 1D), again the PLP thymus showed a significantly higher relative organ weight at this age (Figure 1E, $P < 0.01$).

Protein expression of PCNA, SIRT1 and p53

PCNA protein expression in 21 day old PLP and recuperated thymus was significantly ($P < 0.001$) higher than that in control tissues (Figure 2A). However, at 12 weeks of age the PCNA expression in PLP thymus tended to be higher than controls ($P = 0.07$), while the PCNA protein level in 12 week old recuperated thymus was significantly lower than controls ($P < 0.05$) (Figure 2B). Similar to our findings for PCNA protein levels, SIRT1 protein expression in 21 day old PLP and recuperated thymus was also significantly higher than controls (Figure 2C; $P < 0.05$ for PLP and $P < 0.01$ for recuperated). At 12 weeks of age SIRT1 protein expression in PLP thymus remained higher compared to controls ($P < 0.05$); in contrast, SIRT1 protein expression in recuperated thymus at this age was similar to that of controls (Figure 2D). Examination of the intrathymic level of p53 protein revealed that there was significantly a higher level in the thymus from recuperated mice at both 21 days and 12 weeks of age compared to controls (Figure 2E and 2F). In contrast, there was no significant difference in p53 protein level between PLP and control thymus at these two time points (Figure 2E and 2F).

Concentrations of sex steroid hormones

Although the exact mechanisms that initiate age-associated thymic involution are currently unknown, several studies have shown that sex hormones can influence involution [23]. We therefore determined the serum concentration of testosterone and estradiol in the three animal groups at both ages. At 21 days of age all three groups of mice had similar concentrations of testosterone. A marked increase in testosterone concentration was observed in all animals at 12 weeks of age. However, the magnitude of this increase appeared to differ between the three groups: control mice showed a 15-fold increase (from 0.4 nM to 6 nM), PLP mice showed 7.6-fold increase (from 0.5 nM to 3.8 nM) and recuperated animals an 8.8-fold increase (from 0.5 to 4.4 nM). Thus testosterone concentration in 12 week old PLP and recuperated mice was 37% and 27%, respectively, lower than that of controls. Measuring serum concentrations of estradiol in these three animal groups at different ages, revealed that the estradiol concentration (34 pM) in PLP

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090429

252 mice at 21 days of age was 20% lower than that of controls (43 pM) whereas the
253 concentration in recuperated animals was similar to those of control mice. However, by
254 12 weeks of age the estradiol concentration was similar between the three animal groups.

255 **Gene expression analysis in 21 day old thymus**

256 The above findings suggest that maternal nutrition can alter thymic size and cellular
257 division, moreover given that these animals have varying lifespan, these observations also
258 imply that the early life events that affect longevity, which we have identified using this
259 animal model, may also influences the aging of the thymus. To gain further insight into
260 the possible mechanisms that are likely to impact on the aging of the thymus, we
261 examined the levels of mRNA expression of various genes that are associated with
262 thymic growth and function [13,23-25] in 21 day old thymus from all three animal groups.

263 (1) *Genes involved in the cell cycle:* Cyclin D1 and the cyclin dependent kinase
264 inhibitors p21 and p27 mRNA levels showed no difference between the three groups
265 (Figure 3A-C). The intrathymic expression of p16 mRNA was similar in recuperated and
266 control animals. In contrast, the expression of p16 in PLP thymus was significantly
267 reduced ($P < 0.001$) in comparison to both control and recuperated thymi (Figure 3D).

268 (2) *Genes for sex hormone receptors:* Androgen receptor (AR) expression in PLP
269 thymus was significantly lower than controls ($P < 0.01$) whereas the expression level in
270 recuperated thymus was comparable to controls (Figure 4A). Transcripts for the
271 intracellular estrogen receptor α (ER α) showed no significant differences between the
272 three groups (Figure 4B). However, membrane estrogen receptor GPR30 expression in
273 both PLP and recuperated thymus were significantly increased compared to the controls
274 ($P < 0.05$) (Figure 4C).

275 (3) *Genes involved in thymic growth and aging:* Transcripts for both prothymic
276 interleukin-7 (IL-7) and its receptor IL-7R showed higher levels of expression in
277 recuperated thymus compared to controls ($P < 0.05$ for IL-7 and $P < 0.01$ for IL-7R).
278 Expression of these genes in PLP thymus was similar to controls (Figure 5A and 5B).
279 Thymosin α 1 and thymosin β 4 showed no significant changes in PLP and recuperated
280 thymus compared to controls (Figure 5C and 5D). TGF β 2 receptor gene expression was
281 also not significantly different between the 3 groups (Figure 5E). Rwd1 is a recently
282 characterized thymus aging related protein that has been suggested to be indirectly
283 involved in androgen receptor signaling [26]. The expression level of Rwd1 in PLP and
284 recuperated thymus were similar to controls (Figure 5F). Transcripts of the thymocyte
285 cell surface markers CD4 and CD8 both showed significantly higher level of expression
286 in recuperated thymus whereas in PLP thymus only CD8 showed significantly higher
287 expression levels than controls (Figure 5G and 5H).

Discussion

288

289

290 The thymus is responsible for the generation of immunocompetent T cells which are an
291 integral part of the immune system and therefore this organ plays an important role in
292 functional immunity. The development of the thymus takes place during the late
293 fetal/early postnatal stages of mammalian growth [27] and as such may be sensitive to
294 early life events. Indeed, there is evidence to suggest that the thymus may be affected by
295 early-life nutrition [11,28,29]. We therefore hypothesized that maternal diet may
296 influence thymic development which, in turn may play a role in mediating programming
297 effects on the regulation of lifespan in our maternal protein restricted animals. The
298 present study monitored thymic weight and protein/gene expression of three groups of
299 mice (control, PLP and recuperated) at 21 days and 12 weeks of age. It revealed that
300 while thymi from control and recuperated mice showed no weight gain between 21 days
301 and 12 weeks of age, PLP mice showed significant growth between these two time points.
302 As a result thymic weight of PLP mice was significantly heavier than that of controls at
303 12 weeks of age.

304

305

306

307

308

309

310

311

312

313

314

315

316

317

318

319

320

321

322

323

324

325

326

327

328

329

330

331

332

333

The differential thymic growth in these three groups of mice was apparently related to different mitotic activities in the thymic tissues. Firstly, PCNA protein expression was higher in PLP and recuperated mice at 21 days of age compared to the same age controls. PCNA was originally identified as an antigen that is expressed in the nuclei of cells during the S-phase of the cell cycle and is a cofactor of DNA polymerase essential for DNA replication [30]. This may suggest that cell proliferation in the thymus was enhanced in both PLP and recuperated animals at weaning. However, the enhanced thymic mitotic activity in these two groups may occur due to disparate underlying mechanisms. In the PLP thymus this may be due to selective tissue growth or metabolic resource re-allocation in order to spare vital organ growth whereas in recuperated mice it may be a result of general catch-up growth. This proposal is supported by the fact that only PLP thymus showed a significantly higher relative organ weight whereas relative thymus weight of recuperated mice was similar to the controls. At 12 weeks of age, PCNA protein expression in the thymus of PLP mice was comparable to controls, whereas PCNA protein expression in thymus of recuperated mice was decreased compared to controls. This may explain why the thymus from PLP mice showed significant growth between weaning and 12 weeks of age whereas such growth was not observed in recuperated or control mice. Secondly, SIRT1 has been demonstrated to be correlated with cell cycle activity and its progressive loss is associated with cell cycle withdrawal [31]. The significant up-regulation of SIRT1 in the thymus of 21 days old recuperated mice may reflect the general catch-up growth during lactation. Conversely the significant higher SIRT1 expression in PLP thymus at 12 weeks of age could be indicative of higher mitotic activity compared to control and recuperated mice at this age.

It is well documented that thymic growth and involution is influenced by sex steroid hormones [23]. For instance, surgical or chemical castration of aged animals causes regeneration of the thymus, which was reversed by administration of synthetic sex steroids [32]. Furthermore, castrated male mice also showed enhanced thymocyte proliferation [33]. Conversely androgen administration to intact female and castrated male mice leads to increased thymocyte apoptosis [34]. Receptors for androgen, estrogen and progesterone have been identified on both thymocytes and thymic stromal cells,

334 suggesting that steroid sex hormones directly influence both thymic development and
335 involution [23]. We observed that testosterone concentrations in 12 week old PLP and
336 recuperated offspring were lower compared to that of control animals. Furthermore, when
337 androgen receptor mRNA levels were compared at 21 days of age, PLP thymus showed
338 significantly decreased expression compared to controls whereas recuperated mice
339 expressed similar levels to controls. These observations suggest that reduced testosterone
340 exposure through reduced hormone concentration and reduced receptor expression could
341 contribute to the increased post-weaning thymic growth in PLP animals. Estradiol
342 concentrations were reduced in 21 day old PLP mice. Estrogen functions through
343 intracellular receptors (ER- α and ER- β) as well as a membrane receptor (GPR30), which
344 are all expressed in immune cells. It has been suggested that ER- α and GPR30 mediate
345 the functions of estrogens on the thymus whereas ER- β is not thought to play a role in
346 thymus function [35]. ER- α expression in the thymus at 21 days of age showed no
347 difference between control, PLP and recuperated mice. mRNA levels of GPR30 in the
348 thymus from PLP and recuperated mice were higher than in control tissues. Thus it is
349 possible that increased RPR30 expression in recuperated animals may result in increased
350 estrogen signaling leading to thymic atrophy whereas reduced estradiol concentrations in
351 PLP mice may counteract this effect and favor thymic growth.

352 We recently observed that rats born to low protein fed dams that underwent
353 postnatal catch-up growth showed accelerated telomere shortening in organs including
354 aorta [36] and pancreatic islets [37] which might result from an increased oxidative stress.
355 Thus it is possible that rapid catch-up growth during lactation results in increased
356 oxidative stress in tissues leading to premature cellular aging. Thymus from recuperated
357 mice had significantly elevated p53 protein levels at 21 days and 12 weeks of age. As p53
358 is up-regulated in response to oxidative stress [38], this may suggest that oxidative stress
359 is elevated in recuperated thymus and may contribute to reduced mitotic activity and
360 accelerated aging of the thymus in these animals. Indeed the expression level of PCNA in
361 12 week old recuperated thymus was significantly lower than 12 week old controls.
362 Expression analysis of genes involved in cell cycle regulation showed no significant
363 differences in cyclin D1, p21 and p27 in the thymus between control, PLP and
364 recuperated mice, suggesting that these genes are not primarily responsible for the
365 observed differences in thymic growth patterns in the three groups.

366 Reduced expression of p16 in PLP thymus at 21 days of age may indicate that the
367 thymus in PLP mice undergoes delayed immunosenescence leading to extended lifespan
368 in these animals. p16 is a cyclin-dependent kinase inhibitor that causes cell cycle arrest. It
369 is up-regulated in senescent cells [38] and has been identified as a biomarker/effector of
370 aging [39]. It has been implicated in the age-related decline in regenerative capacity of
371 haematopoietic stem cells [40], pancreatic islets [41] and neural stem cells [42].
372 Suggestion that increased expression of p16 is causally implicated in senescence and
373 organismal aging is supported by studies showing that inactivation of p16 delays cellular
374 senescence and aging in a murine progeria model [43]. p16 expression in pretransplant
375 renal biopsies is inversely related to transplant outcome providing a novel marker
376 predicting postoperative organ function [44]. The current findings suggest that PLP mice
377 demonstrate increased mitotic activity and consequently growth during early adult life
378 whereas recuperated mice may have a thymus that experiences enhanced oxidative stress
379 which can lead to accelerated involution later in life. This is in line with the observations

380 in humans that prenatal nutritional conditions have a long-term impact on thymic
381 function [11,28,29].

382 Intrathymic expression of IL-7 and IL-7 receptor is essential for thymic growth
383 and development of the immune system. IL-7 functions through its receptor to promote
384 thymocyte proliferation and survival [45]. It has been demonstrated that a decline in IL-7
385 expression is associated with age-related thymic atrophy and administration of IL-7 to
386 older mice augments thymic size and thymopoiesis [46]. Increased expression of IL-7 and
387 its receptor in recuperated thymus at 21 days of age is thus consistent with the finding
388 that thymic growth during lactation is enhanced in the recuperated animals. Moreover,
389 the significantly increased expression of CD4 and CD8 in recuperated thymus may be
390 indicative of an altered rate of thymocyte maturation in these mice. CD4 and CD8 are
391 thymocyte cell surface markers. Most immature thymocyte progenitors are negative for
392 CD4 and CD8 and maturation of thymocytes occurs during their migration from the
393 thymic cortex to the medulla. This coincides with the differentiation of CD4⁺CD8⁺
394 double-positive immature thymocytes into CD4⁺CD8⁻ and CD4⁻CD8⁺ single-positive
395 mature thymocytes [16].

396 Thymosin peptides are thymic-derived hormones that have been shown to decline
397 with age and are associated with thymic involution and immune senescence [16]. No
398 significant differences in expression of thymosin α 1 and thymosin β 4 in 21 day old
399 control, PLP and recuperated thymi suggests that these molecules are not involved in the
400 regulation of differential thymic growth observed in this study. Similarly, because Rwd1
401 showed no difference in expression between the three groups it is unlikely that this
402 molecule contributes to this regulation. Transforming growth factor β 2 (TGF- β 2) is
403 known to play an age-dependent negative role in controlling thymic weight and
404 cellularity [47]. Our observation that TGF β RII showed no significant difference between
405 the three groups may suggest that fluctuation in TGF- β 2 receptor expression is not a
406 factor mediating the effects of maternal diet on thymic growth in early postnatal life.

407 In summary, the current findings demonstrate that maternal protein restriction
408 influences thymic growth during young adult life. Offspring of maternally protein
409 restricted mice during lactation preserve thymic growth despite whole body growth being
410 significantly slowed. More importantly the thymus in these mice showed significant
411 further growth during young adult life such that at 12 weeks of age the thymus weight
412 was significantly higher than that of control animals. The underlying causes of this
413 growth appeared to be reduced production of sex steroid hormones, decreased expression
414 of the androgen receptor and increased mitotic activity. In addition, lower expression
415 levels of p16 in PLP thymus at 21 days of age suggests that the aging process in this
416 organ is slowed even at this very early stage of life. In contrast, the thymus in recuperated
417 mice was heavier than controls at 21 days of age which was in line with the whole body
418 catch-up growth. This was associated with increased mitotic activity and increased IL-7
419 and IL-7 receptor expression at this age. Accelerated growth during lactation may result
420 in increased oxidative stress as may be suggested by increased p53 protein expression
421 which could lead to reduced mitotic activity at 12 weeks of age. This may result in
422 accelerated thymic involution which could ultimately contribute to shortened lifespan of
423 recuperated mice. Thus maternal diet and early growth patterns can influence thymic
424 growth and/or involution which may mediate the regulation of lifespan through altered
425 immune functions.

426

Acknowledgments

427

428 We thank K Burling (Core Biochemical Assay Laboratory) for conducting serum
429 testosterone and estradiol measurements and A Wayman, A Flack and D Hawkes for their
430 technical expertise.

431

432

433

Funding

434

435 This work was supported by the Biotechnology and Biological Sciences Research
436 Council [grant number BB/E002161/1], the British Heart Foundation [grant number
437 BS/04/001], the Medical Research Council [grant number G0600717]. SEO is a member
438 of the Medical Research Council Centre for Obesity and Related Metabolic Diseases
439 (MRC CORD), and of the University of Cambridge Metabolic Research Laboratories.

440

441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485

References

- [1] Barker, D.J. and Osmond, C. (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* **1**, 1077-1081.
- [2] McMillen, I.C. and Robinson, J.S. (2005) Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol. Rev.* **85**, 571-633.
- [3] Hanson, M.A. and Gluckman, P.D. (2008) Developmental origins of health and disease: new insights. *Basic Clin. Pharmacol. Toxicol.* **102**, 90-93.
- [4] Gluckman, P.D., Hanson, M.A., Cooper, C. and Thornburg, K.L. (2008) Effect of in utero and early-life conditions on adult health and disease. *N. Engl. J. Med.* **359**, 61-73.
- [5] Martin-Gronert, M.S. and Ozanne, S.E. (2007) Experimental IUGR and later diabetes. *J. Intern. Med.* **261**, 437-452.
- [6] Petry, C.J., Dorling, M.W., Pawlak, D.B., Ozanne, S.E. and Hales, C.N. (2001) Diabetes in old male offspring of rat dams fed a reduced protein diet. *Int. J. Exp. Diabetes Res.* **2**, 139-143.
- [7] Jennings, B.J., Ozanne, S.E., Dorling, M.W. and Hales, C.N. (1999) Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *FEBS Lett.* **448**, 4-8.
- [8] Ozanne, S.E. and Hales, C.N. (2004) Lifespan: catch-up growth and obesity in male mice. *Nature* **427**, 411-412.
- [9] Holt, P.G. and Jones, C.A. (2000) The development of the immune system during pregnancy and early life. *Allergy* **55**, 688-697.
- [10] Calder, P.C., Krauss-Etschmann, S., de Jong, E.C., Dupont, C., Frick, J.S., Frokiaer, H., Heinrich, J., Garn, H., Koletzko, S., Lack, G., Mattelio, G., Renz, H., Sangild, P.T., Schrezenmeir, J., Stulnig, T.M., Thymann, T., Wold, A.E., Koletzko, B. (2006) Early nutrition and immunity - progress and perspectives. *Br. J. Nutr.* **96**, 774-790.
- [11] McDade, T.W., Beck, M.A., Kuzawa, C.W. and Adair, L.S. (2001) Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. *J. Nutr.* **131**, 1225-1231.
- [12] Raqib, R., Alam, D.S., Sarker, P., Ahmad, S.M., Ara, G., Yunus, M., Moore, S.E. and Fuchs, G. (2007) Low birth weight is associated with altered immune function in rural Bangladeshi children: a birth cohort study. *Am. J. Clin. Nutr.* **85**, 845-852.
- [13] Aspinall, R. and Andrew, D. (2000) Thymic atrophy in the mouse is a soluble problem of the thymic environment. *Vaccine* **18**, 1629-1637.
- [14] Aw, D., Silva, A.B., Maddick, M., von Zglinicki, T. and Palmer, D.B. (2008) Architectural changes in the thymus of aging mice. *Aging Cell* **7**, 158-167.
- [15] George, A.J. and Ritter, M.A. (1996) Thymic involution with ageing: obsolescence or good housekeeping? *Immunol. Today* **17**, 267-272.
- [16] Taub, D.D. and Longo, D.L. (2005) Insights into thymic aging and regeneration. *Immunol. Rev.* **205**, 72-93.
- [17] Aw, D., Silva, A.B. and Palmer, D.B. (2007) Immunosenescence: emerging challenges for an ageing population. *Immunology* **120**, 435-446.

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090429

- 486 [18] Pawelec, G., Akbar, A., Caruso, C., Solana, R., Grubeck-Loebenstien, B. and
487 Wikby, A. (2005) Human immunosenescence: is it infectious? *Immunol. Rev.* **205**,
488 257-268.
- 489 [19] Bodey, B., Bodey, B., Jr., Siegel, S.E. and Kaiser, H.E. (1997) Involution of the
490 mammalian thymus, one of the leading regulators of aging. *In Vivo* **11**, 421-440.
- 491 [20] Nikolich-Zugich, J. and Messaoudi, I. (2005) Mice and flies and monkeys too:
492 caloric restriction rejuvenates the aging immune system of non-human primates.
493 *Exp. Gerontol.* **40**, 884-893.
- 494 [21] Vallejo, A.N., Michel, J.J., Bale, L.K., Lemster, B.H., Borghesi, L. and Conover,
495 C.A. (2009) Resistance to age-dependent thymic atrophy in long-lived mice that
496 are deficient in pregnancy-associated plasma protein A. *Proc. Natl. Acad. Sci. U.*
497 *S. A.* **106**, 11252-11257.
- 498 [22] Chen, J.H., Martin-Gronert, M., Tarry-Adkins, J. and Ozanne, S. (2009) Maternal
499 protein restriction affects postnatal growth and the expression of key proteins
500 involved in lifespan regulation in mice. *PLoS ONE* **4**, e4950.
- 501 [23] Hince, M., Sakkal, S., Vlahos, K., Dudakov, J., Boyd, R. and Chidgey, A. (2008)
502 The role of sex steroids and gonadectomy in the control of thymic involution. *Cell.*
503 *Immunol.* **252**, 122-138.
- 504 [24] Rowell, E.A. and Wells, A.D. (2006) The role of cyclin-dependent kinases in T-
505 cell development, proliferation, and function. *Crit. Rev. Immunol.* **26**, 189-212.
- 506 [25] Silva, A.B., Aw, D. and Palmer, D.B. (2009) Neuropeptides and thymic hormones
507 in the *Xenopus* thymus. *Front Biosci.* **14**, 1990-2003.
- 508 [26] Kang, N., Duan, L., Tang, L., Liu, S., Li, C., Li, Y., Liu, Q., Hu, Y., Cui, L., He,
509 W. (2008) Identification and characterization of a novel thymus aging related
510 protein Rwd1. *Cell. Mol. Immunol.* **5**, 279-285.
- 511 [27] Anderson, G. and Jenkinson, E.J. (2001) Lymphostromal interactions in thymic
512 development and function. *Nat. Rev. Immunol.* **1**, 31-40.
- 513 [28] Collinson, A.C., Moore, S.E., Cole, T.J. and Prentice, A.M. (2003) Birth season
514 and environmental influences on patterns of thymic growth in rural Gambian
515 infants. *Acta Paediatr.* **92**, 1014-1020.
- 516 [29] Moore, S.E., Prentice, A.M., Wagatsuma, Y., Fulford, A.J., Collinson, A.C.,
517 Raqib, R., Vahter, M., Persson, L.A., Arifeen, S.E. (2009) Early-life nutritional
518 and environmental determinants of thymic size in infants born in rural Bangladesh.
519 *Acta Paediatr.* **98**, 1168-1175.
- 520 [30] Prosperi, E. (1997) Multiple roles of the proliferating cell nuclear antigen: DNA
521 replication, repair and cell cycle control. *Prog. Cell Cycle Res.* **3**, 193-210.
- 522 [31] Sasaki, T., Maier, B., Bartke, A. and Scoble, H. (2006) Progressive loss of SIRT1
523 with cell cycle withdrawal. *Aging Cell* **5**, 413-422.
- 524 [32] Greenstein, B.D., Fitzpatrick, F.T., Adcock, I.M., Kendall, M.D. and Wheeler,
525 M.J. (1986) Reappearance of the thymus in old rats after orchidectomy: inhibition
526 of regeneration by testosterone. *J. Endocrinol.* **110**, 417-422.
- 527 [33] Olsen, N.J., Viselli, S.M., Shults, K., Stelzer, G. and Kovacs, W.J. (1994)
528 Induction of immature thymocyte proliferation after castration of normal male
529 mice. *Endocrinology* **134**, 107-113.
- 530 [34] Olsen, N.J., Viselli, S.M., Fan, J. and Kovacs, W.J. (1998) Androgens accelerate
531 thymocyte apoptosis. *Endocrinology* **139**, 748-752.

- 532 [35] Wang, C., Dehghani, B., Magrisso, I.J., Rick, E.A., Bonhomme, E., Cody, D.B.,
533 Elenich, L.A., Subramanian, S., Murphy, S.J., Kelly, M.J., Rosenbaum, J.S.,
534 Vandembark, A.A., Offner, H. (2008) GPR30 contributes to estrogen-induced
535 thymic atrophy. *Mol. Endocrinol.* **22**, 636-648.
- 536 [36] Tarry-Adkins, J.L., Martin-Gronert, M.S., Chen, J.H., Cripps, R.L. and Ozanne,
537 S.E. (2008) Maternal diet influences DNA damage, aortic telomere length,
538 oxidative stress, and antioxidant defense capacity in rats. *Faseb J.* **22**, 2037-2044.
- 539 [37] Tarry-Adkins, J.L., Chen, J.H., Smith, N.S., Jones, R.H., Cherif, H. and Ozanne,
540 S.E. (2009) Poor maternal nutrition followed by accelerated postnatal growth
541 leads to telomere shortening and increased markers of cell senescence in rat islets.
542 *Faseb J.* **23**, 1521-1528.
- 543 [38] Chen, J.H., Stoeber, K., Kingsbury, S., Ozanne, S.E., Williams, G.H. and Hales,
544 C.N. (2004) Loss of proliferative capacity and induction of senescence in
545 oxidatively stressed human fibroblasts. *J. Biol. Chem.* **279**, 49439-49446.
- 546 [39] Krishnamurthy, J., Torrice, C., Ramsey, M.R., Kovalev, G.I., Al-Regaiey, K., Su,
547 L. and Sharpless, N.E. (2004) Ink4a/Arf expression is a biomarker of aging. *J.*
548 *Clin. Invest.* **114**, 1299-1307.
- 549 [40] Janzen, V., Forkert, R., Fleming, H.E., Saito, Y., Waring, M.T., Dombkowski,
550 D.M., Cheng, T., DePinho, R.A., Sharpless, N.E., Scadden, D.T. (2006) Stem-cell
551 ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* **443**,
552 421-426.
- 553 [41] Krishnamurthy, J., Ramsey, M.R., Ligon, K.L., Torrice, C., Koh, A., Bonner-Weir,
554 S. and Sharpless, N.E. (2006) p16INK4a induces an age-dependent decline in islet
555 regenerative potential. *Nature* **443**, 453-457.
- 556 [42] Molofsky, A.V., Slutsky, S.G., Joseph, N.M., He, S., Pardal, R., Krishnamurthy,
557 J., Sharpless, N.E. and Morrison, S.J. (2006) Increasing p16INK4a expression
558 decreases forebrain progenitors and neurogenesis during ageing. *Nature* **443**, 448-
559 452.
- 560 [43] Baker, D.J., Perez-Terzic, C., Jin, F., Pitel, K., Niederländer, N.J., Jeganathan, K.,
561 Yamada, S., Reyes, S., Rowe, L., Hiddinga, H.J., Eberhardt, N.L., Terzic, A., van
562 Deursen, J.M. (2008) Opposing roles for p16Ink4a and p19Arf in senescence and
563 ageing caused by BubR1 insufficiency. *Nat. Cell Biol.* **10**, 825-836.
- 564 [44] McGlynn, L.M., Stevenson, K., Lamb, K., Zino, S., Brown, M., Prina, A.,
565 Kingsmore, D. and Shiels, P.G. (2009) Cellular senescence in pretransplant renal
566 biopsies predicts postoperative organ function. *Aging Cell* **8**, 45-51.
- 567 [45] Aspinall, R. (2006) T cell development, ageing and Interleukin-7. *Mech. Ageing*
568 *Dev.* **127**, 572-578.
- 569 [46] Andrew, D. and Aspinall, R. (2002) Age-associated thymic atrophy is linked to a
570 decline in IL-7 production. *Exp. Gerontol.* **37**, 455-463.
- 571 [47] Hsu, H.C., Li, L., Zhang, H.G. and Mountz, J.D. (2005) Genetic regulation of
572 thymic involution. *Mech. Ageing Dev.* **126**, 87-97.
- 573
- 574

575

Figure legends

576

577 **Figure 1. Effects of maternal protein restriction on thymic weights between 21 days**
578 **and 12 weeks of age.** (A) Thymus weights at 21 days and 12 weeks of age, (B) body
579 weights at 21 days of age, (C) relative thymus weight at 21 days of age, (D) body weight
580 at 12 weeks of age and (E) relative thymus weight at 12 weeks of age. Data are expressed
581 as mean \pm SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (for 21 days of age, n = control (C):
582 10, postnatal low protein (PLP): 8, recuperated (R): 10; for 12 weeks of age, n = C: 9,
583 PLP: 9, R: 9).

584

585 **Figure 2. Effects of maternal protein restriction on PCNA, SIRT1 and p53 protein**
586 **levels in thymus at 21 days and 12 weeks of age.** (A) Relative PCNA protein levels in
587 thymus at 21 days of age, (B) relative PCNA protein levels at 12 weeks of age, (C)
588 relative SIRT1 protein levels at 21 days of age, (D) relative SIRT1 protein levels at 12
589 weeks of age, (E) relative p53 protein levels at 21 days of age and (F) relative p53 protein
590 levels at 12 weeks of age. Protein expression levels were measured by Western blotting
591 followed by densitometry analysis. Data are expressed as mean \pm SE with mean protein
592 levels in thymus from control mice being set as 100. * $P < 0.05$, ** $P < 0.01$, *** $P <$
593 0.001 compared to control (n = 8 for each group).

594

595 **Figure 3. Effects of maternal protein restriction on relative gene expression of cyclin**
596 **D1 (A), p21 (B), p27 (C), and p16 (D) in thymus at 21 days of age.** Gene expression
597 levels were measured by quantitative RT-PCR. Data are expressed as mean \pm SE with
598 mean gene expression in thymus from control mice being set as 100. *** $P < 0.001$ (n = 8
599 for each group).

600

601 **Figure 4. Effects of maternal protein restriction on relative gene expression of AR**
602 **(A), ER- α (B) and GPR30 (C) in thymus at 21 days of age.** Gene expression levels
603 were measured by quantitative RT-PCR. Data are expressed as mean \pm SE with mean
604 gene expression in thymus from control mice being set as 100. * $P < 0.05$, ** $P < 0.01$,
605 *** $P < 0.001$ (n = 8 for each group). AR: androgen receptor; ER- α : estrogen receptor α ;
606 GPR30: G protein-coupled membrane receptor for estrogens.

607

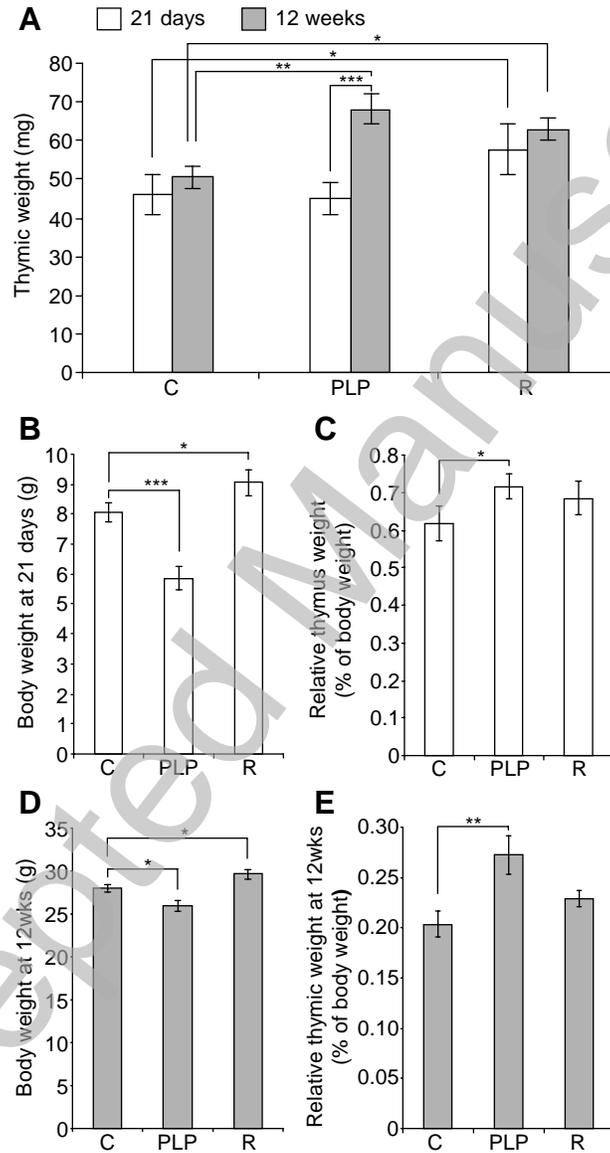
608 **Figure 5. Effects of maternal protein restriction on relative gene expression of IL-7**
609 **(A), IL-7 receptor (B), Thymosin α 1 (C), Thymosin β 4 (D), TGF β RII (E), Rvdd1 (F),**
610 **CD4 (G) and CD8 (H) in thymus at 21 days of age.** Gene expression levels were
611 measured by quantitative RT-PCR. Data are expressed as mean \pm SE with mean gene
612 expression in thymus from control mice being set as 100. * $P < 0.05$, ** $P < 0.01$, *** $P <$
613 0.001 compared to control (n = 8 for each group).

614 **Table 1.** Primer sequences for genes investigated in this study
615

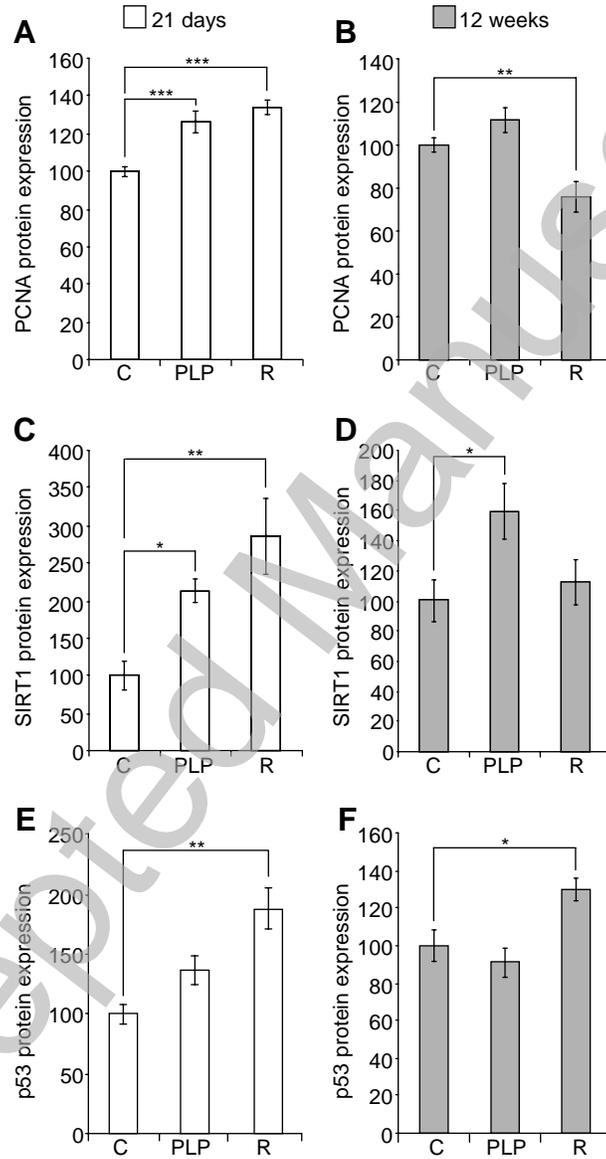
616		Forward primers	Reverse primers	Amplicon (bp)
617				
618	P16	CTTTGTGTACCGCTGGGAAC	GCCGGATTTAGCTCTGCTCT	111
619	P21	TTGCACTCTGGTGTCTGAGC	TCTGCGCTTGGAGTGATAGA	112
620	P27	CGCCAGACGTAAACAGCTC	TCAATGGAGTCAGCGATATG	120
621	Cyclin-D1	GAGTCTCCGGTGCATCATT	CTTCGCAAAGGCAGAGTCTT	101
622	AR	CTCACCAAGCTCCTGGATTC	GAAAGTCCACGCTCACCATA	100
623	ER α	GACCAGAATTGGGTCATGAGA	AGAGACCAAGACCCAGAGG	97
624	GPR30	CCATAGCCTACGTGCCAGA	TCCACGTAGCAGGCATGTAA	91
625	CD4	TGCACGCTGAAATCCTACAA	CCTTGGGTAGTCATCAACCA	99
626	CD8	ATGCTCTTGGCTCTTCCAGA	GC'TTCTCGTCCCACGTTATC	101
627	Thymosin α 1	GAGGAAGAGGAGGAGGAGGA	ATCCTCAGCTACCCGCTTG	99
628	Thymosin β 4	AACGCAAGAGAAAAATCCTCTG	GCCAGCTTGCTTCTCTTGTT	61
629	IL-7	AGGCACACAAACACTGGTGA	TCTCTTAGGAAACATGCATCA	88
630	IL-7R	CCTCCTATGTGTGGAGACACC	AGGTAGGGCACTCATGTGG	110
631	Rwdd1	AGAGGAGCAAGCAGGGAAA	ATCCACCTCCACATTGTTCC	118
632	TGF β RII	GTAAGGATGGGCTGCCAGT	GGCTTTGTGTTGCTTCCCTA	93
633	β -Actin	TTGCTGACAGGATGCAGAAG	CCACCGATCCACACAGAGTA	103
634				

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090429

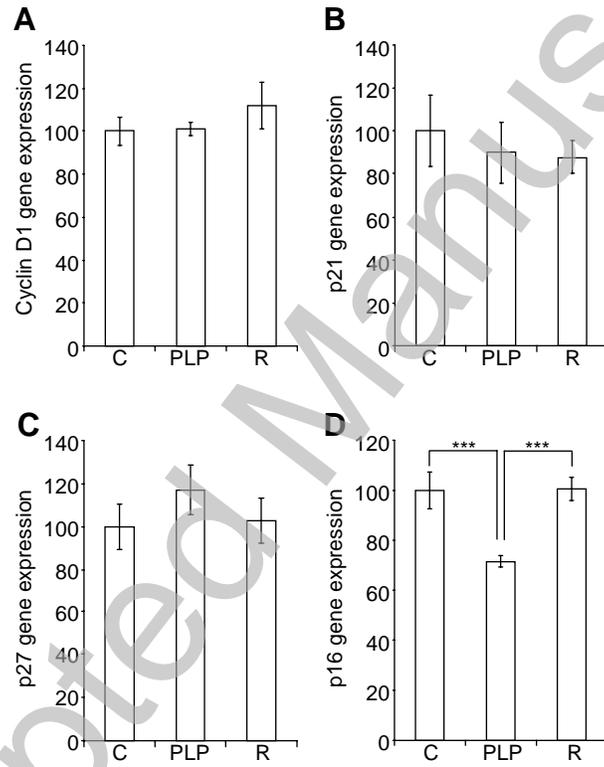
Accepted Manuscript



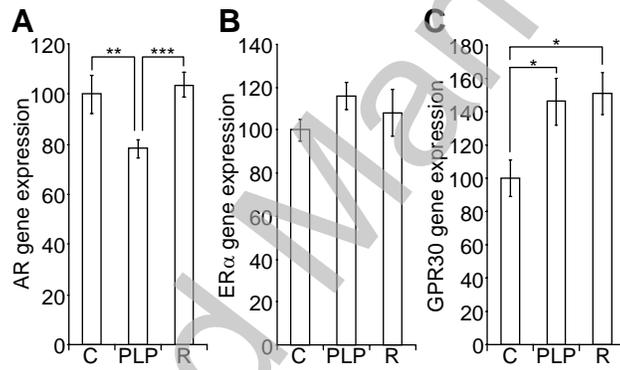
Chen_et_al Figure 1



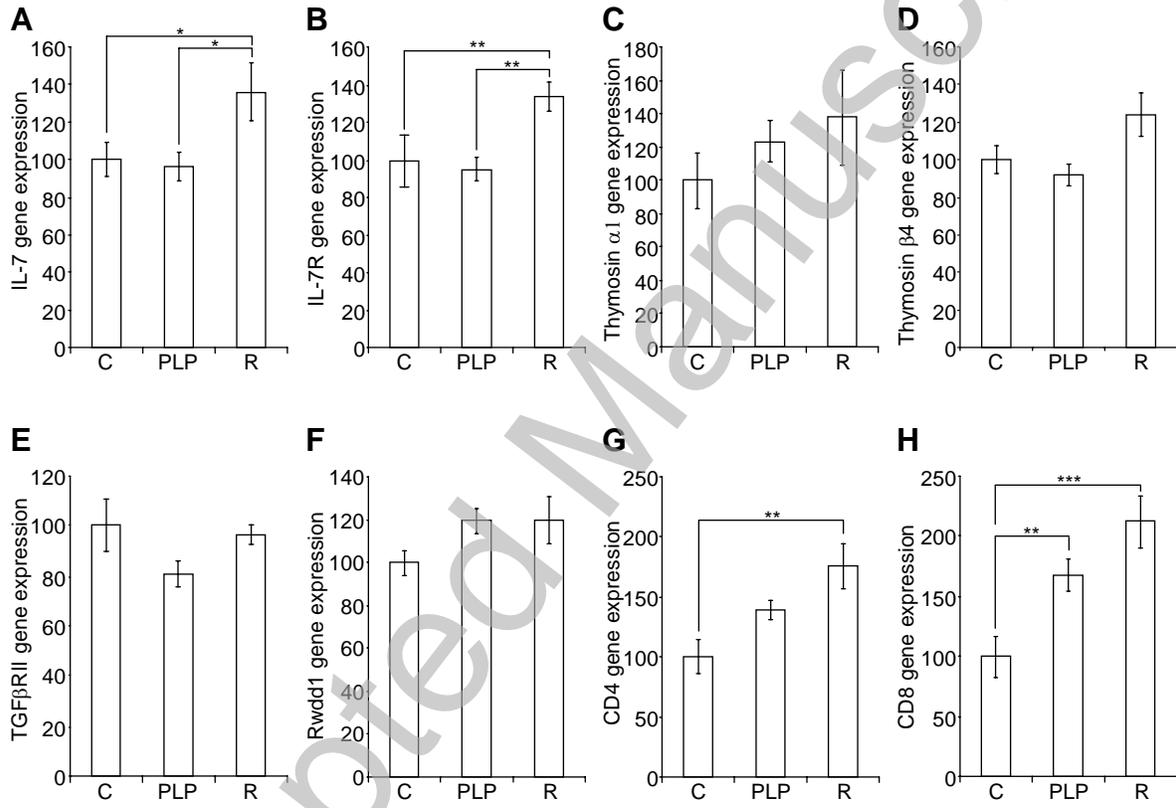
Chen_et_al Figure 2



Chen_et_al Figure 3



Chen_et_al Figure 4



Chen_et_al Figure 5