**Leptin and Leptin receptor polymorphisms in infants and their parents: correlation with preterm birth**

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## Abstract

It has been proved that Single Nucleotide Polymorphisms (SNPs) in LEP and LEPR genes could predispose an increased risk of pregnancy adverse outcomes (PAOs) as recurrent pregnancy loss (RPL) and pre-eclampsia. Preterm birth (PTB) is the leading cause of infant mortality. We decided to investigate the correlation between PTB and LEP and LEPR SNPs.

The study cohort included families who underwent spontaneous PTB and control samples of families who had at term (≥37 weeks of gestational age) born children. Swabs were performed by

rubbing the sticky end for about 30 seconds on the gum and on the inside of the cheek, allowing to collect the flaking cells of the oral mucosa. Genotyping of the three SNPs, LEPR A668G, LEP G2548A and A19G, was carried out by ARMS-MAMA real-time PCR procedure previously described.

Regarding LEPG2548A, we found that the most expressed genotype in infants both in preterm and at term group was AG, however we did not discover any statistically significant difference (p = 0.97). Considering LEPA19G, none among infants and parents resulted to carry AA genotype. No statistically significant differences were found between children, mothers and fathers belonging to preterm and at term group. We did not find a statistically significant association in newborns and their mother, but our results show a statistical correlation with the LEPRA668G genotype GG of the father. This fact can contribute to define genetic risk factors for PTB. Further studies are certainly needed to better clarify the role of genetics in influencing preterm delivery.

## Keywords

Leptin, leptin receptor, polymorphisms, preterm birth, infants, genetics, parents, buccal swab

## 1. Introduction

To date, leptin (LEP), a polypeptide hormone made of 167 amino acids, is mainly known for its role in regulating hungry and satiety mechanisms [1, 2]. However, numerous scientific evidences have demonstrated that this adipokine is implicated in the control of many pathways, including bone and cartilage growth, immune system and systemic inflammatory response, acting thus as a "pleiotropic" molecule [3]. What is more, LEP, which is primarily released by adipose tissue but also by placenta [4], seems to play an important role in reproductive functions (such as oocyte maturation, embryo development and placentation) and pregnancy [5]. In fact, it has been shown that serum LEP levels increase significantly during gestation and decrease after birth, demonstrating

an important role of LEP in childbearing [6]. In addition, as described by Gonzalez et al., leptin-deficient mice are infertile and administration of exogenous LEP is able to restore fertility [7]. LEP exerts its actions through its receptor, LEPR, a single transmembrane protein made of 874 amino acids, expressed in the brain and peripheral tissues as kidneys, lungs, stomach, endometrium, placenta and umbilical cord [8, 9]. Not only LEP alterations but also LEPR ones seem to have repercussions on reproduction and pregnancy: Pérez-Pérez et al. theorized that LEP and LEPR anomalies could be implicated in the pathogenesis of recurrent miscarriage, pre-eclampsia and intrauterine growth restriction [4]. It has been proved that "s"ingle "n"ucleotide "p"olymorphisms (SNPs) in LEP and LEPR genes could predispose to these conditions; in particular, LEP *G2548A* (rs7799039) and LEPR A668G (rs1137101) are the most studied SNPs and they have been shown to be associated with an increased risk of pregnancy adverse outcomes (PAOs) as recurrent pregnancy loss (RPL) and pre-eclampsia [10]. Preterm birth (PTB), defined as the birth of a child before 37 completed weeks gestation, is the leading cause of death and disability in children under 5 years of age worldwide [11], but the exact pathogenesis is unknown. Whilst PTB is becoming a preventable condition for a very small subset of women, global rates of PTB continue to rise [12]. Recent estimates report that PTB affects approximately 11% of all livebirths, or approximately 15 million PTB per year [12]. Twin studies have also suggested that genetics account for 17–36% of PTB risk [13]. In attempting to understand the heritability of PTB, both maternal and fetal genetics have been assessed. It has been suggested that maternal genetics are responsible for 22.8% of the variations in gestational age in spontaneous births, with fetal genetics responsible for 12.7% of the variation [13]. About metabolic and biosynthetic Pathway-Related Polymorphisms, sustaining metabolic homeostasis is vital for embryonic development and survival. Polymorphisms within genes responsible for the biosynthesis and metabolism of fatty acids, lipoproteins, triglycerides, and cholesterol have been associated with PTB in women and infants of various ethnicities [14]. Salem.et al., in 2016, investigated the possible correlation between LEP and LEPR SNPs and PTB: they found that women and neonates bearing the homozygous mutated AA form of LEP *G2548A*

genotype had an increased risk of PTB as well as women carrying LEPR A668G AA or AG genotypes had a fourfold increased risk for severe (Gestational Age $\leq$ 32 weeks) PTB [14]. Due to the suggested role of LEP and LEPR in pregnancy and ambiguous results, we decided to investigate the correlation between PTB and LEP and LEPR SNPs. On the basis of implication of SNPs (LEP A19G, rs2167270) in the leptin levels in the first 6 month of life [2] we evaluated also the possible role of a this SNPs on PTB and we decided to extend genetic analysis also to the fathers of the babies; what is more, we performed genetic analysis not on blood but on buccal swab samples and we determined LEP and LEPR polymorphisms through the created by Bergallo et. al procedure of amplification refractory mutation system-mismatch amplification mutation assay (ARMS-MAMA) real-time PCR [15].

## 2. Results

### 2.1.Characteristics of the study population

During the study period 165 maternal, paternal and fetal swab samples were collected. Since one maternal and five paternal samples were lost, genetic analyses were performed on 159 subjects in total. Preterm group accounted for 54 subjects (18 infants + 36 parents), while at term one comprised 35 babies and 70 parents (105 in total). The former group included babies with a gestational age comprised between 26 and 36+6. For each child, an anamnestic form was filled out, registering infant as well as parents' personal data, course of pregnancy, type of delivery and major illness. The main characteristics, in relation to which the two groups resulted homogeneous ($p > 0.05$), of the involved subjects are highlighted in Table 1.

**Table 1.** Demographic description of the studied cohort; values expressed as mean (+/- standard deviation)

| Characterization | Preterm group (n = 54) | At term group (n = 105) | p-value |
|---|---|---|---|
| Gender of newborn % (male) | 38% | 50% | 0.39 |
| Mean maternal age at birth, years | 32 (5.03) | 33.6 (4.19) | 0.26 |
| Mean paternal age at birth, years | 35.6 (4.72) | 39.9 (4.43) | 0.94 |
| Mean gestational age at delivery, weeks | 34.1 (2.87) | 39.3 (2.09) | 0.57 |
| Mean number of pregnancies | 2.28 (1.07) | 1.22 (0.98) | 0.86 |
| Mean number of live birth | 2.43 (1.75) | 1.72 (1.03) | 0.74 |
| Abortions | 0.10 (0.68) | 0.16 (0.93) | 0.93 |
| Caesarean sections | 0.40 (1.02) | 0.25 (0.65) | 0.09 |

### *2.2. Allelic frequencies*

Regarding LEPG2548A, A allele was the most frequent among preterm and at term infants without showing, however, a statistically significant difference ($p = 0.84$); on the other hand, G allele resulted the most common among both mothers and fathers of the two groups with no statistically difference ($p = 0.83$). Considering LEPA19G, G allele appeared to be the most represented among babies and their parents in preterm and at term group; again, the difference was not statistically relevant with a p value of 1 for children, 0.16 for mothers and 0.64 for fathers. At last, we analyzed LEPRA668G allelic frequencies and we found that A allele was the most expressed; however, a not significant difference was found among preterm and at term children as well as among parents (Table 2).

**Table 2.** LEPG2548A, LEPA19G and LEPRA668G allelic frequencies in preterm and at term group.

| SNP | Subjects | Allelic frequencies in preterm group (%) | | Allelic frequencies in at term group (%) | | p-value |
|---|---|---|---|---|---|---|
| | | G | A | G | A | |
| LEP G2548A | Infants | 45 | 55 | 42 | 58 | 0.84 |
| | Mothers | 56 | 44 | 58 | 42 | 0.83 |
| | Fathers | 59 | 41 | 56 | 44 | 0.83 |
| LEP A19G | Infants | 77 | 23 | 76 | 24 | 1.00 |
| | Mothers | 67 | 33 | 79 | 21 | 0.16 |
| | Fathers | 76 | 24 | 71 | 29 | 0.64 |
| LEPR A668G | Infants | 40 | 60 | 33 | 67 | 0.54 |
| | Mothers | 42 | 58 | 39 | 61 | 0.83 |
| | Fathers | 35 | 65 | 27 | 73 | 0.75 |

*2.3. Genotypic frequencies*

Regarding LEPG2548A, we found that the most expressed genotype in infants both in preterm and at term group was AG, however we did not discover any statistically significant difference (p = 97). The mothers of the preterm babies resulted to be mainly carriers of AG genotype, while the ones of at term children had the same frequency of GG and AG genotypes (39%), but no significant difference was discovered among the two groups (p = 0.9) as well as for the fathers (p = 0.95), whose most detected genotype was AG. Considering LEPA19G, none among infants and parents resulted to carry AA genotype. No statistically significant differences were found between children, mothers and fathers belonging to preterm and at term group: preterm and at term infants' most

common genotype was GG, preterm mothers' one was AG while at term ones' was GG; on the other hand, preterm fathers resulted to be mainly carriers of GG genotype and at term ones of AG genotype. Finally, we analyzed LEPRA668G genotypic frequencies and again we did not find any statistically significant result. In preterm babies the most common genotype was AG, in at term ones AA; regarding parents, mothers of the two groups showed both AG genotype as the most frequent, while preterm and at term fathers AA genotype (Table 3).

**Table 3.** LEPG2548A, LEPA19G and LEPRA668G genotypic frequencies in preterm and at term group.

| SNP | Subjects | Genotypic frequencies in preterm group (%) | | | Genotypic frequencies in at term group (%) | | | p-value |
|---|---|---|---|---|---|---|---|---|
| | | GG | AA | AG | GG | AA | AG | |
| LEP G2548A | Infants | 15 | 25 | 60 | 11 | 28 | 61 | 0.97 |
| | Mothers | 33 | 22 | 45 | 39 | 22 | 39 | 0.90 |
| | Fathers | 35 | 18 | 47 | 33 | 21 | 46 | 0.95 |
| LEP A19G | Infants | 55 | - | 45 | 53 | - | 47 | 1.00 |
| | Mothers | 33 | - | 67 | 58 | - | 42 | 0.68 |
| | Fathers | 53 | - | 47 | 42 | - | 58 | 0.55 |
| LEPR A668G | Infants | 15 | 35 | 50 | 12 | 44 | 44 | 0.77 |
| | Mothers | 6 | 27 | 67 | 11 | 28 | 61 | 0.75 |
| | Fathers | 18 | 47 | 35 | 6 | 52 | 42 | 0.42 |

2.4.*Association between neonates and parents polymorphism and preterm births*

We evaluated if there were any differences between mutated homozygotes and wild-type homozygotes + heterozygotes among preterm and at term group for LEPG2548A and LEPRA668G. We not find any statistically significant difference LEPG2548A genotypes analyzed as shown in Table 4. At the contrary we find a statistically significant differences in LEPRA668G genotypes. GG LEPRA668G genotypes in more frequent in fathers of preterm groups. Since none of the subjects resulted to be carrier of mutated homozygous (AA) genotype for LEPA19G, we did not consider LEPA19G in this analysis.

**Table 4**. Mutated homozygous versus (wild-type homozygous + heterozygous) genotypic frequencies for LEPG2548A and LEPRA668G in preterm and at term group.

| SNP | Subjects | Preterm group (%) | | Term group (%) | | p-value |
|---|---|---|---|---|---|---|
| | | AA | AG + GG | AA | AG + GG | |
| LEP G2548A | Infants | 26 | 74 | 28 | 72 | 1.00 |
| | Mothers | 22 | 78 | 22 | 78 | 1.00 |
| | Fathers | 18 | 82 | 21 | 79 | 0.92 |
| | | GG | AG + AA | GG | AG + AA | |
| LEPR A668G | Infants | 16 | 84 | 11 | 89 | 0.75 |
| | Mothers | 11 | 89 | 6 | 94 | 0.31 |
| | Fathers | 18 | 82 | 6 | 94 | *0.0153 |

The father carrying homozygote mutant GG LEPRA668G genotype markedly (Odd ratio 3.6 fold) increase the risk of PTB.

## 3. DISCUSSION

Over the past two decades, there have been significant technological advances that have improved the ability to obtain genetic data and address challenges in disease prevention and treatment. Studies have been conducted to improve the understanding of the biological mechanisms underlying PTB and to translate research findings into a clinical setting. The development of prognostic tools that stratify treatment to enable targeted and personalized PTB prevention has been a major focus of many genetic studies on PTB. PTBs occur at less than 37 weeks' gestational age, accounting for 75% of perinatal mortality. Up to now, preterm birth rate has risen dramatically in most industrialized countries: it is estimated to be around 12-13% in the USA, while 5-9% in Europe [13, 16]. Despite its high incidence, the exact pathogenesis of preterm delivery is still not known: genetic component seems to play a crucial role, particularly variations in maternal genome are thought to be the most implicated in influencing the course of pregnancy; however, recently, it has been proposed that also fetal genetic variations might contribute to childbirth outcome [17, 18]. For this reason, based on the results obtained by Salem et al., we wanted to further investigate the possible impact of LEP and LEPR polymorphisms on preterm delivery [14]. It is, in fact, confirmed that LEP and its receptor are involved in the regulation of reproductive functions as demonstrated by the fact that LEP levels rise significantly during the first gestational trimester along with estrogen and beta-hCG as well as that LEP and LEPR transcripts are expressed early in trophoblast during pregnancy, proving the paracrine and autocrine actions of this adipokine [5, 19, 20, 21]. The association between serum LEP levels and PAOs has been investigated by many researchers and it has been proven that variations in LEP levels significantly increase the risk of PAO, especially the higher the serum LEP values are, the greater the risk of PTB seems to be as theorized by Vàzquez et al. and Strobel et al [22, 23]. LEP and LEPR polymorphisms are known to be associated with altered serum LEP levels: as example, Marcello et al. found that the carriers of homozygote mutant AA genotype for LEPG2548A have higher plasma LEP levels in comparison to carriers of AG and GG genotypes [24, 25]. It is not totally clear through which pathways LEP could lead to PTB,

however it seems that augmented LEP levels are associated with an increase of sympathetic activity, of mitochondrial superoxide synthesis and with an activation of matrix metalloproteinases which modulate the vascular structures; all these conditions are believed to generate oxidative stress as well as endothelial and placental dysfunction [26]. Leptin is a hormone that regulates peripheral energy stores and is therefore mainly found in adipose tissue. It is known that there is a positive correlation between adipose tissue and LEP levels. In pregnant women, LEP levels increase in the first trimester, even before there is a noticeable increase in body weight, suggesting that factors other than increased adiposity influence LEP levels. During pregnancy, the placenta is an additional source of LEP, which also plays a crucial role in cell proliferation, implantation and fetal growth. LEP concentration in cord blood has been found to correlate strongly with placental and birth weight [14]. LEP from the placenta pass into the maternal blood and contribute to maternal LEP blood levels. In fact, most LEP are expressed in the placenta, enter the maternal serum and increase the maternal LEP serum level. Thus, the embryo has a major influence on the LEP serum level of its mother. In this context, our new findings regarding neonatal genetic variation as a factor in PTB are important. Salem et al. show that the effect of LEP polymorphism on PTB is strong and significant and it is sufficient that either the mother or her respected neonate, independently, carry the LEP homozygote mutant AA genotype to markedly increase the risk for PTB [14].

As opposed to Salem et al., we did not find any statistically significant correlation between LEP and LEPR polymorphisms, in infants and mother and PTB. Since LEPG2548A is thought to be in linkage disequilibrium with the less studied LEPA19G, we evaluated also this latter SNP [15, 27, 28]: interestingly none of the subjects resulted to be carrier of the AA genotype, what is more the frequency of the A allele was lower than the one of the G allele in children, mothers and fathers all; given the scarce literature on this SNP, which is mainly studied in relation to neoplastic pathologies, the implication of this data remains to be clarified [29].

We decided to involve in our study also the fathers of the babies since, although childbirth is strictly dependent on the mother-fetus binomial, the genetic material of the embryo derives half from the

paternal contribution [30, 31]. In this contest, considering that Salem et al. found a positive correlation between PTB and the homozygous mutated AA genotype for LEPG2548A in children, we wanted to evaluate the risk of the infant of inheriting the mutated allele from the father and therefore being exposed to an increased probability of PTB [32];

We find a statistically significant difference among preterm and at term group about homozygote mutant LEPRA668G genotype GG reflect in 3.6 fold increase risk of preterm birth.

Also LEPRA668G genotype seem to be involved in plasma levels of leptin [2] and this fact could be associated with oxidative stress as well as endothelial and placental dysfunction [26]. Single nucleotide polymorphisms (SNPs) of LEPR A668G (Q223R, rs1137101) have been certified to affect the binding activity of LEPR and leptin, ultimately changing the leptin signaling. The SNP have been reported to be associated with a variety of diseases, such as type 2 diabetes mellitus (T2DM), polycystic ovary syndrome (PCOS), coronary artery disease, essential hypertension, nonalcoholic fatty liver disease, and endometrial cancer [33]. PTB etiology is multifactor, with a strong genetic component. We don't try any correspondence with previously data reported by Salem but add an actor (Father) in the importance of these LEP and LEPR SNPs.

Regarding the procedure of DNA extraction, we choose to perform it on the less invasive and painful than blood sampling buccal swab. It is, in fact, confirmed that repetitive hurting stimulations in infants, also in the very young ones, can induce changes in the nervous system both at the peripheral and central levels with alteration of the pain threshold [34, 35]. The swab allows the collection of exfoliation cells of the oropharynx and, since the DNA is the same in any cell of an individual, the obtained genetic material can be used in the same way as a blood sample [36]; it has been seen, in fact, that the results of the DNA extraction from different types of biological material are comparable and have the same degree of accuracy [37, 38]. In our experience, this method significantly increased the compliance of the parents in subjecting the child to the procedure of sampling. The main limitations of this study are related to the available sample size, which does not allow generalization of our results; the use of the "late preterm" gestational age of many members

of the preterm group (around 36 weeks), so it might have been more useful for the purposes of the study to consider a population with a lower gestational age to better highlight any differences between the preterm and term groups.

Up to date, it is proven that LEP is implicated in the pathophysiology of many events, even if its function is often underestimated [39]. To the best of our knowledge, this is the second study evaluating a possible correlation between LEP and LEPR polymorphisms and PTB, but the first involving also the fathers of the babies with the aim to investigate the possible role of paternal genetic contribution on delivery outcomes. PTB is a global problem in obstetrics, affecting nearly 15 million babies worldwide every year. The development of PTB is multifactorial and is influenced by both environmental and genetic factors. There is a great deal of research looking at the genetics of PTB, with a focus on identifying the genetic polymorphisms associated with it. Although investigating the genetics of PTB is challenging because the PTB population is highly heterogeneous, we did not find a statistically significant association in newborns and their mother, but our results show a statistical correlation with the LEPRA668G genotype GG of the father. This fact can contribute to define genetic risk factors for PTB. Further studies are certainly needed to better clarify the role of genetics in influencing preterm delivery.

## 4. Materials and methods

### *4.1.Study desig and subjects*

We included in our prospective case-control study 96 infants admitted at "Terapia Subintensiva Allargata Prima Infanzia", University Division, Regina Margherita Children Hospital, Città della Salute e della Scienza di Torino between October 2018 and March 2019. Exclusion criteria were mothers who experienced complicated births or suffered from pre-eclampsia. The study cohort included families who underwent spontaneous PTB and control samples of families who had at term

(≥37 weeks of gestational age) born children. Among the 96 initially included infants, only the parents of 55 children allowed their enrollment in the study with a written informed consent. For each baby, we recruited both father and mother with a total of 165 subjects, who underwent a buccal swab for DNA extraction.

### *4.2.Buccal swab sampling*

Swabs were performed by rubbing the sticky end for about 30 seconds on the gum and on the inside of the cheek, allowing to collect the flaking cells of the oral mucosa; they were then placed in a special test tube, without liquid or culture medium, and stored in the refrigerator ( at – 80 C°) before the extraction procedure.

### *4.3.DNA extraction and genotyping description*

Genomic DNA was extracted from swab using the Maxwell16 LEV Blood DNA kit with automatic extractor Maxwell16 System (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions. Genotyping of the three SNPs, LEPR A668G, LEP G2548A and A19G, was carried out by ARMS-MAMA real-time PCR procedure previously described by Authors [15]. These assays have been the subject of technology transfer in favor of the academic spin-off of the University of Turin BioMole (BioMole srl. Turin, Italy) G2548A-LEP rs7799039 PP-BioMole-033, G19A-LEP rs2167270 PP-BioMole-034 and Q223R-LEPR rs1137101 PP-BioMole-035 and they are now commercially available. In the ARMS-PCR assay, the specificity of forward or reverse primers is given by the terminal 3′ nucleotide, while the MAMA-PCR improves the discrimination power of the allele-specific primers by adding a mismatched nucleotide near the 3′ terminal region. Therefore, the ARMS-MAMA method allows to greatly reduce the background noise and it is, thus, a reliable and easily interpretable technique, without needing to process the post- amplification sample as occurred, instead, in the PCR-RFLP (Restriction Fragment Length Polymorphism) procedure.

*4.4.Statistical analysis*

Data analysis were conducted on the total sample, using the statistical application GraphPad Prism (version 5). Sample size was evaluated by power analysis. Demographic data as well as information on pregnancy, delivery and the neonatal period were presented as descriptive statistics. Continuous variables were expressed as the means and standard deviations. For each SNP, the allele frequencies were computed by simple counting and the Hardy-Weinberg equilibrium was tested by Fisher’s exact test. Bivariate analyzes to compare categorical variables were performed using Fisher's and Chi-square statistical tests, while independent samples t-test to compare quantitative variables. All tests were done in two sails, with a fixed significance alpha = 5% ($p = 0.05$).

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**Informed Consent Statement**: Informed consent was obtained from all subjects involved in the study.

**Conflicts of Interest**: The authors declare no conflict of interest

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