ACYLATED/UNACYLATED GHRELIN RATIO IN CORD BLOOD: CORRELATION WITH ANTHROPOMETRIC AND METABOLIC PARAMETERS AND PEDIATRIC LIFESPAN COMPARISON

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Abstract

Context. Ghrelin is a peptide with multiple functions that circulates as acylated (AG) and non acylated (UAG) forms. The role of ghrelin in neonates remains to be clarified.

Objective. The aim of the study was to determine ghrelin concentrations of the two forms in neonates in order to clarify their biological roles. As such, ghrelin levels at birth were compared with those in later life.

Setting and Design. Tertiary Care Center. In this cross-sectional study we evaluated AG, UAG, AG/UAG ratio and insulin levels in venous cord blood from neonates (NN) and in fasted normal weight (NW) and obese (OB) children, both prepubertal and pubertal.

Subjects. We studied 82 Neon, 82 NW and 58 OB children.

Results. AG levels were lower in Neon than NW and OB children (p<0.0001), more specifically the prepubertal NW and OB children (p<0.0001). UAG levels were higher in NN than in NW and OB children (p<0.0001). Therefore, the AG/UAG ratio was lower in NN than NW and OB children (p<0.0001). NN showed insulin levels similar to NW and lower than OB children (p<0.0001). At birth UAG was positively correlated with AG (Pearson: 0.425; p<0.0001) and negatively with insulin (-0.253; p<0.02). In NW and OB, UAG and AG were positively correlated to each other and negatively correlated with insulin and BMI (-0.566; p<0.0001).

Conclusions. NN when compared to children, showed higher UAG and lower AG levels. The AG/UAG ratio showed a very different profile in NN, being lower than in NW and OB children, thus suggesting a different metabolic function for the two forms for the neonatal age. Further studies are needed to clarify the exact role of the different ghrelin forms in neonates.
Introduction

Ghrelin is a 28 amino-acid peptide predominantly secreted by the stomach, but also by the hypothalamus and the placenta in rats and humans (1). Ghrelin is characterized by a strong GH-releasing activity mediated by the activation of the GH Secretagogue (GHS) receptors (GHS-R) and has stimulatory effect on PRL and ACTH secretion (2). Ghrelin has also orexigenic effects and induces adiposity (3). The secretion occurs in a pulsatile manner and levels change throughout the day with especially high levels before food intake and during the night. There is a reduction in ghrelin immediately after feeding, suggesting that it plays a role in meal initiation (4). Hyperglycemia and insulin decrease plasma ghrelin levels (5). Conversely, total ghrelin induces hyperglycemia and decreases plasma insulin concentrations (6).

Ghrelin circulates as two forms, acylated (AG) and non acylated (UAG). The acylation of the peptide consists of an octanoyl group bound to serine-3 residue which seems critical for the binding to the GHS-receptor type 1a, GH-releasing activity and the other endocrine actions (7). However, UAG, which circulates at far higher levels than AG, is not biologically inactive because it is able to exert metabolic, cardiovascular and antiproliferative endocrine effects, probably by binding to different GHS-receptor subtypes or other receptor families (8-9). In particular, both AG and UAG seem to play an important regulatory role in metabolism. In humans, AG induces a rapid rise in glucose and insulin levels when administrated alone. UAG co-administration counteracts this effect (9).

The role of ghrelin, as it pertains to growth in the neonatal period, is still poorly defined. Ghrelin is found in human fetal circulation during gestation. Small for gestational age (SGA) newborns show ghrelin levels higher than in adequate (AGA) or large for gestational age (LGA) neonates (10-11). The most shared hypothesis is that ghrelin could have an anabolic role at the beginning of meals and in energy balance, however there is limited evidence to support this theory (4).
In childhood, ghrelin regulation and secretion are also not fully understood. Ghrelin levels are increased in anorexia nervosa, reduced in obesity and restored by weight recovery. A small fall in ghrelin levels with age over childhood from prepuberty to puberty has been observed (1). This most likely can be explained by the participation of sexual hormones in ghrelin regulation (12).

Regarding the two forms of ghrelin, UAG seems to be lower and therefore the AG/UAG ratio is higher in obese children with and without metabolic syndrome when compared to normal weight children (13,14). Instead, children that fail to thrive, show AG and total ghrelin levels more elevated than control subjects. This is thought to be an adaptive mechanism to increase appetite and preserve energy balance (15). In neonates, UAG appears higher in SGA compared to AGA neonates (10), with no differences in AG levels (16). To date, the role of AG and UAG in neonates and children is unclear and poorly studied, particularly at birth.

We hypothesize that, at birth, AGA neonates show ghrelin levels similar to prepubertal children, with the same AG/UAG ratio. In order to understand the lifespan regulation and the biological implications of the two ghrelin forms at the neonatal age, we evaluated AG and UAG levels at birth compared with those of normal weight and obese children, both prepubertal or pubertal.

**Subjects and Methods**

We studied 3 groups of consecutive Caucasian subjects: neonates (NN), normal weight (NW) and obese (OB) children according to Italian growth charts (17-18).

Group NN was composed of 82 caucasian neonates (40 males and 42 females), born to term (37-41 weeks of gestation) and AGA with a normal ponderal index. AGA was defined as a birth weight from 10th to 90th percentile for gestational age according to Italian charts (18). All babies were born after uncomplicated pregnancies and were otherwise healthy. Thirty-eight were born by vaginal delivery and 44 from caesarean delivery. All the mothers were healthy and in
particular none of the mothers had gestational diabetes. None of the babies showed signs of
distress at delivery. Birth weight and length were recorded at birth by the attending nurse.
Group NW included 82 children (46 females and 36 males) born AGA. Of these 44 were
prepubertal and 38 with a pubertal stage from II to V according to Tanner scale (19-20). ). NW
subjects have their weight included between 3rd and 75th percentile of Italian Charts (17). These
children presented to the clinic for an evaluation of growth, pubertal status, suspected thyroid
disease, general health check-up, but no disease was confirmed at the end of the evaluations.
Group OB was composed by 58 children (30 females and 28 males), 28 of which were prepubertal
and 30 pubertal. All OB children were born AGA. All NW and OB children were randomly
enrolled according to the clinical criteria at Division of Pediatrics, University of Piemonte
Orientale, Novara, Italy. The cohort population is the same described in the manuscript in press. In
that paper NW and obese children have been punctually analysed discussing differences in ghrelin
levels between the two groups, evaluating the influence of pubertal status and of metabolic pattern.
In the present manuscript the attention is focused on neonates while children have been used as a
comparison population.
Exclusion criteria were the presence of any psychiatric or organic diseases in particular
neurological, endocrine (short stature), liver and kidney abnormalities. None of the children were
under pharmacological treatments. The study protocol was approved by an Independent Ethical
Committee and the informed consent was obtained from each child’s parents.
All auxological parameters of the three groups are reported in Table 1.
Height was measured by the Harpenden stadiometer and weight by using electronic scale. Body
mass index (BMI) was calculated as body weight divided by squared height (kg/m²). Rohrer’s
ponderal index was calculated as body weight divided by cube length (kg/m³). Puberty was
assessed exclusively by two trained physicians following pubertal Tanner stages. In females,
puberty was considered when the breasts appeared, in males when testicular volume was greater than 4 ml.

In cord blood at birth in NN, and at 8.30-9.00 hours following an overnight fasting in NW and OB plasma, we measured AG, UAG and insulin. The AG/UAG ratio was calculated. In NN, at delivery, the cord was immediately clamped and venous blood samples were drawn by catheterization.

Human ghrelin (fmol/ml) was measured in acidified plasma stored at -80C using ELISA kits DRG Instruments GmbH, Marburg, Germany. AG: sensitivity: 1 fmol/ml. Intra- and inter-assay CV ranges: 3.5-3.8% and 2.6-3.9% UAG: sensitivity 10 fmol/ml Intra e inter-assay CV ranges: 2.1-4.7 % and 4.2-7.2%.

Insulin (µUI/ml; 1µUI/ml = 7.175 pmol/l) was measured by chemiluminescent enzyme-labelled immunometric assay (Diagnostic Products Corporation, Los Angeles, CA). Sensitivity: 2 µUI/ml. Intra- and inter-assay CV ranges: 2.5-8.3 and 4.4-8.6%.

Data are expressed as mean ± SEM. Distributions of continuous variables were examined for skewness and were logarithmically transformed, where appropriate. Differences between groups were assessed using Student’s t-test or one-way ANOVA with post hoc analysis using Bonferroni test. A correlation analysis was performed using the Pearson’s correlation test. Statistical significance was assumed for p<0.05. All statistical analyses were performed with SPSS for Windows version 15.0 (SPSS INC; Chicago, IL, USA).

Results

AG levels (mean ± SEM) were lower in NN when compared to both NW (1.68±0.24 vs 8.43±0.87 fmol/ml; p<0.0001) and OB children (1.68±0.24 vs 5.30±0.68 fmol/ml; p<0.0001) [Fig.1]. AG levels were particularly lower in NN than in prepubertal NW and OB children (9.77±1.06 and 6.23±0.73 fmol/ml, respectively; p<0.007). UAG levels were higher in NN
(213.2±9.1 fmol/ml) when compared with NW (135.9±8.7 fmol/ml; p<0.0001) and OB children (79.5±7.6 fmol/ml; p<0.0001) [Fig.1] (Table 2).

Furthermore, AG/UAG ratio was lower in NN than in NW (0.01±0.0 vs 0.07±0.01; p<0.0001) and OB children (0.01±0.0 vs 0.07±0.01; p<0.0001). AG/UAG ratio was similar between NW and OB without pubertal differences [Fig.1].

NN showed insulin levels (6.40±0.76 µUI/ml) similar to NW (6.26±0.51 µUI/ml) and lower than OB children (14.40±1.24 µUI/ml; p<0.0001).

AG, UAG levels and the AG/UAG ratio were not different in NN according to the type of delivery. No gender differences were detected in each of the three groups.

At birth UAG was positively correlated with AG (Pearson: 0.425; p<0.0001) and negatively with insulin (-0.253; p<0.02). No association was found between UAG and anthropometric parameters. AG did not demonstrate any associations with anthropometric or hormonal parameters, with the exception of UAG. In NW and OB, UAG was positively correlated with AG (0.537; p<0.0001) and negatively with insulin and BMI (-0.566 and -0.541; p<0.0001). Similarly, AG was positively correlated with UAG and negatively with insulin and BMI (-0.442 and -0.323; p<0.0001).

In a model composed of all 3 groups, UAG was negatively correlated with weight and insulin (β:-0.661 and -0.489, respectively; p<0.0001) and AG was weakly associated in a negative manner exclusively with insulin (β:-0.214; p<0.003).

**Discussion**

Our study is mainly focused on a physiological investigation of the two forms of ghrelin, AG and UAG, in healthy AGA newborns compared to later in life. The results demonstrate that full term neonates in venous cord blood at birth present a very different profile of the two ghrelin forms compared to that found in children. Neonates show lower AG and higher UAG levels than normal
weight and obese children, independent of pubertal status. As a consequence, the AG/UAG ratio in cord blood of neonates is lower when compared to that found in NW and OB children.

To date, most authors have studied total ghrelin independent of the two forms in neonates and children (12, 21-26), with a few studies published regarding the ghrelin isoforms, particularly in newborns.

It has been clearly demonstrated that total ghrelin levels are similar in female and male newborns (12, 16, 27, 28) and are higher in SGA compared to AGA newborns (10, 24, 25), while controversial data exist regarding correlations between ghrelin levels and gestational age or auxological parameters (16, 25, 29, 30). Soriano-Guillen et al (12) demonstrated that total ghrelin levels in newborns were similar between full-term and preterm, increasing during early postnatal life and decreasing thereafter during puberty with a negative correlation between ghrelin, age and Tanner stages.

Only a few studies have shown that AG is present in fetal and neonatal circulation (16, 27, 30) equally between preterm and SGA newborns, and full term and AGA, without differences with respect to gender. Moreover, no correlations were found between AG and auxological parameters (16, 27, 31). Recently Mendez-Ramirez et al. (10) measured UAG levels in AGA and SGA newborns at the age of one week of life, showing that UAG was higher in SGA compared than AGA neonates. To date, no authors have studied UAG levels in cord blood.

In our study we opted to use an assay based on a double-antibody sandwich technique where a monoclonal antibody specific to the C-terminus of ghrelin is coated to the multi-well plate and detection is performed using an acetylcholinesterase labelled antibody specific to the N-terminus of ghrelin, therefore sandwiching AG when present. This ELISA kit has been demonstrated to have a greater assay specificity, particularly with respect to nutritional states (32). Using the same assay, we have previously discussed data related to AG and UAG in prepubertal and pubertal NW and OB children (33). In the present study, our data demonstrates that the AG/UAG ratio is very different in
the venous cord blood of neonates compared to later in life, demonstrating lower AG and higher UAG levels than normal weight and obese children. Interestingly AG/UAG ghrelin ratio is lower in neonates than in children, considering the prepubertal age and pubertal age. This is supported by studies in rat embryos, where elevated plasma concentrations of UAG and lower AG were demonstrated, with a circulating AG/UAG ratio that increased from fetal day 20 to postnatal days (34). A possible hypothesis is that UAG levels could be higher at birth, reflecting the fetal state, due to the immaturity of the GOAT system that turns non acylated ghrelin into the acylated form. This enzyme has been recently discovered to be responsible for ghrelin octanoylation, but its physiological role and regulation is at present unclear, particularly in the fetal state and childhood. Furthermore, the placenta has been demonstrated to express very low levels of the GOAT transcript (35, 36).

Some authors have described the regulation of UAG and AG with respect to metabolic impairments in adulthood. Rodriguez et al. demonstrated that obese subjects with respect to lean individuals had increased levels of AG and decreased UAG (37). Barazzoni et al. (14) demonstrated that AG/UAG ratio in patients with metabolic syndrome was increased and positively correlated with insulin resistance indexes compared to non obese subjects. Pacifico et al. (13) showed lower UAG levels and higher AG/UAG ratio in patients with metabolic syndrome than in those without metabolic syndrome. Therefore, at present, the available information seems to suggest that pathological conditions may likely influence ghrelin form levels and their ratio.

In the literature, acute AG administration in adult subjects induced a rapid increase in glucose and insulin levels with AG related to insulin resistance. On the contrary, UAG prevented AG effects when coadministered with AG and its levels have been found to be negatively associated with insulin levels and insulin resistance (8-9). Also in our study UAG levels and insulin showed a negative correlation, suggesting a major metabolic implication of UAG rather than AG in the neonatal period. Taking into account data in the literature together with our data, we can speculate
that the peculiar state of ghrelin secretion in venous cord blood and the negative correlation between UAG and insulin levels, is focused to improve insulin sensitivity in the fetal state. Therefore, at birth, UAG could have a different role with respects to AG. Our data strengthens the importance of the different AG/UAG ratio, proposing a role in metabolic function and fetal growth. Accordingly, neonates showed insulin levels similar to NW and lower than in obese children. Insulin levels primarily contribute to neonatal growth as insulin is one of its major hormone regulators promoting lipogenesis, glycogenesis and protein synthesis (38).

There is a high degree of controversy regarding the relationship between ghrelin and anthropometric parameters. A negative association between UAG and birth weight has been demonstrated by Mendez-Ramirez et al (10) suggesting that diminished body weight induces different adaptative signals. A recent study of Martos-Moreno et al. (16) assessing both preterm and term newborns, failed, like us, to demonstrate any association between AG and anthropometric indices, including ponderal index. Our study is in line with the majority of studies failing to find an association at birth, even if it has to be considered that our population include only AGA neonates. Moreover, both forms of ghrelin were independent of gender. The data in the literature are concordant with these results in neonates (16, 27). The type of delivery does not influence ghrelin levels in our study nor in the literature (21, 30, 10).

In conclusion, our study demonstrated that in physiological conditions, neonates show higher UAG and lower AG levels compared to children in later life, resulting in a lower AG/UAG ratio. This hormonal pattern and the negative correlation between UAG and insulin levels would suggest a different metabolic function at birth. These peculiarities could be related to rapid hormonal and metabolic changes that could influence weight gain in early postnatal life. As such, it is important that further studies be performed to clarify the exact role of different ghrelin forms in fetal and postnatal life.
Declaration of interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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34. Chanoine JP & Wong AC. Ghrelin gene expression is markedly higher in fetal pancreas compared with fetal stomach: effect of maternal fasting. Endocrinology 2004 145 3813-20


Figure 1. Plasma acylated ghrelin (AG, fmol/ml), unacylated ghrelin (UAG, fmol/ml) and acylated/unacylated ghrelin ratio (AG/UAG ratio), in neonates (black bar) at birth and in normal weight (NW) and obese (OB) children (white bars). Data are expressed as mean ± SEM. Differences between groups were assessed using one-way ANOVA with post hoc analysis using Bonferroni test. * = p<0.0001
Tab. 1 Clinical parameters of neonates, normal weight and obese children.

<table>
<thead>
<tr>
<th></th>
<th>Neon</th>
<th>NW</th>
<th>OB</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>All PP</td>
<td>P</td>
</tr>
<tr>
<td>n</td>
<td>82</td>
<td>82</td>
<td>44</td>
</tr>
<tr>
<td>M/F</td>
<td>40/42</td>
<td>36/46</td>
<td>26/18</td>
</tr>
<tr>
<td>EU/TC</td>
<td>37/45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GA (wk)</td>
<td>38.9±0.18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PI (kg/m$^3$)</td>
<td>2.6±0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>9.7±0.47</td>
<td>7.99±0.42</td>
<td>14.49±0.56</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>3.28±0.06</td>
<td>31.44±1.6 $^b$</td>
<td>25.86±1.17 $^a$</td>
</tr>
<tr>
<td>c° weight</td>
<td>50.15±2.88</td>
<td>39±3.4</td>
<td>39.2±3.86</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>50±0.31</td>
<td>129.7±2.48 $^b$</td>
<td>121.6±2.26 $^a$</td>
</tr>
<tr>
<td>c° height</td>
<td>49.8±0.31</td>
<td>33±3.6</td>
<td>32.9±4.13</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>-</td>
<td>17.67±0.35 $^b$</td>
<td>16.96±0.35 $^a$</td>
</tr>
<tr>
<td>c° BMI</td>
<td>-</td>
<td>39±3.10</td>
<td>38.8±3.51</td>
</tr>
</tbody>
</table>

Abbreviations: c°: percentile; EU: eutocical delivery; GA: gestational age; Neon: neonates; NW: normal weight; OB: obese; PI: ponderal index; PP: prepubertal; P: pubertal; TC: caesarean delivery. a: p<0.0001 NW and OB PP vs P; b: p<0.0001 NW vs OB.
Tab. 2 Hormonal parameters of neonates, normal weight and obese children.

<table>
<thead>
<tr>
<th></th>
<th>Neon</th>
<th>NW</th>
<th>OB</th>
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<tbody>
<tr>
<td></td>
<td>All</td>
<td>PP</td>
<td>P</td>
</tr>
<tr>
<td>AG (fmol/ml)</td>
<td>1.68±0.24&lt;sup&gt;ab,c&lt;/sup&gt;</td>
<td>8.43±0.87&lt;sup&gt;ae&lt;/sup&gt;</td>
<td>9.77±1.06&lt;sup&gt;bf&lt;/sup&gt;</td>
</tr>
<tr>
<td>UAG (fmol/ml)</td>
<td>213.2±9.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>135.9±8.7&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>155.8±10.2&lt;sup&gt;bf&lt;/sup&gt;</td>
</tr>
<tr>
<td>AG/UAG ratio</td>
<td>0.01±0.00&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.07±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: AG: acylated ghrelin; Neon: neonates; NW: normal weight; OB: obese; PP: prepubertal; P: pubertal; TC: caesarean delivery; UAG: non acylated ghrelin. a: <0.0001 N vs NW plus OB; b: p<0.0001 Neon vs each group (NW/OB PP/P); c: p<0.01 Neon vs each group (NW/OB PP/P); d: p<0.0001 NW vs OB; e: p<0.01 NW vs OB; f: p<0.0001 NW and OB PP vs P.
Figure 1.

267x179mm (600 x 600 DPI)