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 in acylated (AG) and unacylated (UAG) forms. However, the role of ghrelin in neonates (NN) remains to be clarified.

Objective: The aim of this study was to determine ghrelin concentrations of the two forms in NN 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 NN and in fasted normal weight (NW) and obese (OB) children, both prepubertal and pubertal.

Subjects: We studied 82 NN, 82 NW, and 58 OB children.

Results: AG levels were lower in NN than in 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 in 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 body mass index (−0.566; P<0.0001).

Conclusions: NN compared with 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 in NN. Further studies are needed to clarify the exact role of the different ghrelin forms in NN.

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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 prolactin 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 in two forms, acylated (AG) and unacylated (UAG). The acylation of the peptide consists of an octanoyl group bound to serine-3 residue that seems critical for the binding to the GHS-R 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-R 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 administered 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 for gestational age (AGA) or large for gestational age neonates (LGA) (10, 11). The widely held 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 (OB) children with and without metabolic syndrome compared with normal weight (NW) 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 (NN), UAG appears higher in SGA compared with AGA NN (10), with no differences in AG levels (16). To date, the role of AG and UAG in NN and children is unclear and poorly studied, particularly at birth.

We hypothesize that, at birth, AGA NN show ghrelin levels similar to prepubertal children, with the same AG/UAG ratio. 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 NW and OB children, both prepubertal and pubertal.

**Materials and methods**

We studied three groups of consecutive Caucasian subjects: NN, NW, and OB children according to Italian growth charts (17, 18).

Group NN was composed of 82 Caucasian NN (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 the 10th to the 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 cesarean 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 in a pubertal stage from II to V according to Tanner scale (19, 20). NW subjects have their weight included between the 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 checkup, but no disease was confirmed at the end of the evaluations.

Group OB was composed of 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 have been described elsewhere (21). In that paper NW and OB children had been punctually analyzed 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 NN 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 Ethics 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 electronic scale. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m²). Rohrer’s ponderal index was calculated as body weight divided by length cubed (kg/m³). Puberty was assessed exclusively by two trained physicians following pubertal Tanner stages. In females, puberty was ascertained by the appearance of breasts and, in males when testicular volume was > 4 ml.

In cord blood at birth in NN, and at 0830–0900 h following an overnight fast 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 catherization.

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

Insulin (µIU/ml; 1 µIU/ml = 7.175 pmol/l) was measured by chemiluminescent enzyme-labeled immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Sensitivity: 2 µIU/ml intra- and inter-assay CV ranges: 2.5–8.3 and 4.4–8.6%.

Data are expressed as mean ± s.e.m. Distributions of continuous variables were examined for skewness and were logarithmically transformed, where appropriate. Differences between the groups were assessed by the
In NW and OB, UAG was positively correlated with AG or hormonal parameters, with the exception of UAG. Not demonstrated any associations with anthropometric 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).

Results

AG levels (mean ± S.E.M.) were lower in NN compared with 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) 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 and 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 μIU/ml) similar to NW (6.26 ± 0.51 μIU/ml) and lower than OB children (14.40 ± 1.24 μIU/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).

Discussion

Our study is mainly focused on a physiological investigation of the two forms of ghrelin, AG and UAG, in healthy AGA newborns compared with later in life. The results demonstrate that in full-term NN, the venous cord blood at birth presents a very different profile of the two ghrelin forms compared with that found in children. NN show lower AG and higher UAG.

**Table 1**Clinical parameters of NN, NW, and OB children.

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<th>NN</th>
<th>NW</th>
<th>OB</th>
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<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>
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<tr>
<td>GA (week)</td>
<td>38.9 ± 0.18</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PI (kg/m²)</td>
<td>2.6 ± 0.3</td>
<td>–</td>
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| Age (years) | 9.7 ± 0.47  | 7.99 ± 0.42* | 14.49 ± 0.56* |
| Weight (kg)  | 3.28 ± 0.06  | 31.44 ± 1.17† | 25.86 ± 1.17† |
| Per, weight | 50.15 ± 2.88 | 39.2 ± 3.86  | 37.3 ± 7.3   |
| Height (cm)  | 50 ± 0.31    | 129.7 ± 2.48† | 121.6 ± 2.26† |
| Per, height | 49.8 ± 0.31  | 33.6 ± 3.6   | 32.9 ± 4.13 |
| BMI (kg/m²)  | –           | 17.67 ± 0.35† | 16.96 ± 0.35† |
| Per, BMI    | 39 ± 3.10    | 38.8 ± 3.51  | 39.5 ± 6.8  |

Per, percentile; EU, eutocical delivery; TC, cesarean delivery; GA, gestational age; PI, ponderal index; PP, prepubertal; P, pubertal. *P < 0.0001 NW and OB PP vs P; †P < 0.0001 NW vs OB.

**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 ± S.E.M. Differences between the groups were assessed by the one-way ANOVA with post-hoc analysis by the Bonferroni test. *P < 0.0001.
levels than NW and OB children, independent of pubertal status. As a consequence, the AG/UAG ratio in cord blood of NN is lower compared with that found in NW and OB children.

To date, most authors have studied total ghrelin independent of the two forms in NN and children (12, 22–27), 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, 28, 29) and are higher in SGA compared with AGA newborns (10, 25, 26), while controversial data exists regarding correlations between ghrelin levels and gestational age or auxological parameters (16, 26, 30, 31). 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, 28, 31) 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, 28, 32). Recently, Mendez-Ramirez et al. (10) measured UAG levels in AGA and SGA newborns at the age of 1 week of life, showing that UAG was higher in SGA compared with AGA NN. 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 onto the multiwell plate and detection is performed by an acetylcholinesterase labeled antibody specific to the N-terminus of ghrelin, therefore sandwiching AG when present. This ELISA kit has been demonstrated to have greater assay specificity, particularly with respect to nutritional states (33). Using the same assay, we have previously discussed data related to AG and UAG in prepubertal and pubertal NW and OB children (21). In this study, our data demonstrates that the AG/UAG ratio is very different in the venous cord blood of NN compared with later in life, demonstrating lower AG and higher UAG levels than NW and OB children. Interestingly, AG/UAG ghrelin ratio is lower in NN than in children, considering the prepuberal 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 UAG into the AG form. This enzyme has recently been 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. (37) demonstrated that OB subjects with respect to lean individuals had increased levels of AG and decreased UAG. Barazzoni et al. (14) demonstrated that AG/UAG ratio in patients with metabolic syndrome was increased and positively correlated with insulin resistance indexes compared with non-OB 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 co-administered 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 respect to AG. Our data strengthens the importance of the different AG/UAG ratio, proposing a role in metabolic function and fetal growth.

**Table 2** Hormonal parameters of NN, NW, and OB children.

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<th>NN</th>
<th>NW</th>
<th>OB</th>
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<tr>
<td></td>
<td>All</td>
<td>PP</td>
<td>P</td>
</tr>
<tr>
<td>AG (fmol/ml)</td>
<td>1.68±0.24±3±1</td>
<td>8.43±0.87±3±1</td>
<td>9.77±1.06±1±1</td>
</tr>
<tr>
<td>UAG (fmol/ml)</td>
<td>213.2±9.1±1</td>
<td>135.9±8.7±5</td>
<td>155.8±10.2±1</td>
</tr>
<tr>
<td>AG/UAG ratio</td>
<td>0.01±0.00±1±1</td>
<td>0.07±0.01*</td>
<td>0.07±0.01±1</td>
</tr>
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</table>

AG, acylated ghrelin; PP, prepubertal; P, pubertal; UAG, non-acylated ghrelin. *P<0.0001 NN vs NW plus OB; †P<0.0001 NN vs each group (NW/OB PP/P); ‡P<0.01 NN vs each group (NW/OB PP/P); ††P<0.0001 NN vs OB; ‡‡P<0.01 NN vs OB; ¶P<0.0001 NW and OB PP vs P.
Accordingly, NN showed insulin levels similar to NW and lower than in OB 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 adaptive signals. A recent study by 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 includes only AGA NN. Moreover, both forms of ghrelin were independent of gender. The data in the literature are concordant with these results in NN (16, 28). The type of delivery does not influence ghrelin levels in our study nor in the literature (21, 31, 10).

In conclusion, our study demonstrated that in physiological conditions, NN show higher UAG and lower AG levels compared with 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

The 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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