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Research Article

Predictive Value of Ghrelin Levels in the Cord Blood of Preterm Infants

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Abstract

Introduction: Ghrelin enables fetal organ growth and maturation.

Objective: Since ghrelin controls growth hormone and insulin-like growth factor-1 secretion in utero, we theorized umbilical blood concentrations of ghrelin correlates with fetal growth and organ-specific disease. Hence, we studied umbilical blood levels of ghrelin as predictive of postnatal diseases in preterm infants born at ≤ 32 weeks of gestation.

Methods: We compared ghrelin levels in cord blood with outcomes among preterm infants born at ≤ 32 weeks of gestation that did or did not have diseases of prematurity and healthy term infants. An immunoassay measured the concentrations of ghrelin in umbilical blood.

Results: Ghrelin did not predict fetal growth but a regression model identified a weak but significant relationship between low ghrelin concentrations in umbilical blood and the occurrence of Stage I - II ROP (p = .02). While not statistically significant, infants > 28 wks with ghrelin levels < 40 pg/mL at birth were more likely to have stage I and II retinopathy of prematurity (OR = 2.4), Stage III ROP (OR = 3.8), and IVH Grade 3 - 4 IVH (OR = 3.7).

Conclusion: Ghrelin content in umbilical cord blood may identify the risk of disease in preterm infants and deserves more research in conjunction with other biomarkers.

Keywords: *Angiogenesis; Cord Blood; Ghrelin Concentrations; Inflammation; Preterm Infants; Retinopathy of Prematurity*

Abbreviations

ANOVA: Analysis of Variance; BPD: Bronchopulmonary Dysplasia (Chronic Lung Disease of Prematurity); hGH: Human Growth Hormone; IGF-1: Insulin-Like Growth Factor-1; IVH: Intraventricular Hemorrhage; IL: Interleukin (+ # Per Family Member); NEC: Necrotising Enterocolitis; NFκB: Nuclear Factor Kappa B; NICU: Neonatal Intensive Care Unit; PDA: Patent Ductus Arteriosus; ROP: Retinopathy of Prematurity; SGA: Small for Gestational Age; TNF: Tumour Necrosis Factor; VEGF: Vascular Endothelial Growth Factor

Introduction

Hormones and growth factors are crucial to fetal tissues because they modulate growth, maturation, and metabolism [1,2]. While researchers have mostly focused on insulin and IGF-1, the placenta expresses ghrelin and this 28-amino acid peptide is present in amniotic fluid and fetal blood [3]. Thesecretagogue is necessary not only for the development of the placenta, but it also affects fetal growth and

maturation [4]. Foetuses swallow amniotic fluid and this physiology is essential for third trimester nutrition [5]. Ghrelin participates in regulation of swallowing via the fetal/neonatal hypothalamic-pituitary-adrenal axis [6]. Particularly in very preterm infants, the lungs and gastrointestinal tract are critical to transition to life outside the uterus. In foetuses, ghrelin is a mediator of pulmonary growth and development [7] and intestinal maturation [8]. Docosahexaenoic acid reduces the severity of ROP by inhibiting NFκB [9]. Research found ghrelin blocks development of ROP because of its indirect regulation of the release of IGF-1 and VEGF [10]. Ghrelin also blocks NFκB activation in endothelium [11]. Based on the preceding evidence, we hypothesized that concentrations of ghrelin in umbilical blood relates to gestational age and anthropometric measures at birth. Moreover, proposed ghrelin may be a potential predictor of diseases after preterm birth.

Methods

Participants

The Bioethical Committee of Warsaw Medical University approved this research investigation on May 26, 2009. The study required written consent from parents who wanted their neonates to participate in the study. Study participants were neonates admitted to the Department of Neonatology and the NICU of the Medical University of Warsaw from January 2010 through December 2012. The Department of Neonatology provides care after delivery of sick and healthy premature and term infants.

Inclusion criteria were 1) preterm infants with a gestational age ≤ 32 wks with and without illness, 2) healthy infants born at term (gestational age ≥ 37 wks) and 3) parental consent for participation in the study. Exclusion criteria were no major congenital malformations, chromosomal abnormalities, syndromes or associations, and/or metabolic disorders. A prospective enrolment of subjects took place at this hospital, and researchers assigned them to one of three groups:

- **Group A** (n = 27) were premature infants born at ≤ 32 weeks of gestational age who had one or more of the following diseases: BPD, IVH, NEC, PDA and ROP. Among infants in Group a (sick preterm), we used published criteria to classify a hemodynamically significant PDA [12], IVH [13], NEC [14], ROP [15] and BPD [16]. In this group, the occurrence of investigated illness was PDA requiring medical therapy or surgical ligation, stage I, II and stage III ROP, grade 3 and 4 IVH, NEC (≥ stage 2), and BPD requiring O_2 therapy at 36 weeks post-conceptual age.
- **Group B** (n = 32) were well appearing preterm infants after birth at ≤ 32 weeks of gestation, but following a screening cranial ultrasound infants were identified with haemorrhages.
- **Group C** (n = 27) were term infants born at ≥ 37 weeks of gestation (n = 27) that had no disease manifest during hospitalization.

We sought correlations between ghrelin levels in cord blood and the following clinical findings:

Birthweight and length	Bronchopulmonary dysplasia
Occipital frontal circumference	Duration of non-invasive ventilation
Gender	Duration of mechanical ventilation
Gestational age	Duration of oxygen therapy
Type of delivery	Retinopathy of prematurity - stage
Apgar scores at 1 and 5 minutes	Intraventricular haemorrhage - grade
Mortality	Necrotizing Enterocolitis

Ghrelin Assay

Investigators recovered a two mL sample of cord blood after birth, followed by centrifugation of the specimen and storage of the plasma in Eppendorf™ tubes at -23°C until analysed. The cord blood concentrations of ghrelin were measured using an immunoassay

(DRG Human Ghrelin total - EIA 4709; Biocompare, Poznañ, Poland) and was performed in the radioimmunology laboratory at the Memorial Child Health Centre Institute in Warsaw, Poland. The analytical range was 0 to 1600 pg/mL. The intra- and inter-assay variability was < 10%.

Statistical Methods

Investigators based sample size calculations on the recommendations of G*Power statistical software (www.gpower.hhu.de/en.html), and for a multiple regression with 16 predictors, an effect size f^2 0.5, a power of .80 and an alpha of .05, a minimum of 54 total subjects were required. Descriptive statistics included medians with IQR as well as means ± standard deviation for each group. We assessed normality using the Shapiro-Wilk's test ($p > 0.05$). If the values were normally distributed, we used an independent t-test for a two-group comparison and a one-way ANOVA for comparing the three groups of infants. We assessed homogeneity of variances by the Levene's test. If the values were not normally distributed, we performed non-parametric analysis using the Mann-Whitney U test for two groups and the Kruskal-Wallis test for three groups. We used the Chi-square test for independence to measure the association of nominal level variables. We used multiple regression to evaluate the relationship of neonatal variables (outcomes) in preterm infants (< 37 wks post-conceptual age) with their umbilical blood ghrelin levels. During analyses, we ascertained assumptions of linearity, independence of errors, homoscedasticity, unusual points and normality of residuals. We presented measures of clinical significance as odds ratios (ORs) and 95% confidence intervals (CIs). Level of significance was set at $p \leq 0.05$. Statistical analyses utilized SPSS version 23 (IBM, New York City, New York).

Results

Table 1 shows the demographics of the three groups of study infants. The occurrence of diseases of prematurity in Group A (sick preterm) were a hemodynamically significant PDA = 11/27 (four ligations), Stage I or II ROP = 15/27, stage III ROP = 5/27, grade 3 or 4 IVH = 3/27, NEC = 4/27, and BPD = 10/27. Group B infants had no ROP of any stage on retinal examination, but 17 of 32 infants screened by cranial ultrasonography had a subependymal or a grade 2 IVH. One death occurred in the entire study population; it occurred in Group A. Gestational age and birthweight were not normally distributed between the sick preterm (Group A)and well preterm (Group B) infants as assessed by the Shapiro-Wilk's test ($p < 0.05$). A Mann-Whitney U test was run to determine if there were differences in gestational age and birth weight between Group A and Group B. Gestational age was statistically significantly higher in Group B (median = 30 wks) versus Group A (median = 29 wks), $p < 0.001$. Birth weight was also higher in Group B (median = 1555g) compared to Group A (median = 1055g), $p < 0.001$. We found that ghrelin levels were not normally distributed for all three groups as assessed by the Shapiro-Wilk's test ($p < 0.05$). As such, we performed a Kruskal-Wallis test to determine if there were differences in the cord blood ghrelin among the three groups. Table 2 shows means and median ghrelin levels were not statistically significantly different among the three groups ($p > 0.05$). Umbilical blood ghrelin measurements in SGA Infants compared to AGA infants was not different (median = 250 versus 238 pg/mL, respectively, $p = 0.85$). Gender did not influence the levels of ghrelin in umbilical blood among the three groups (female median = 240 vs. male median = 238 pg/mL, $p = 0.62$).

Identifying Factors	A Preterm Infants-postnatal disease	B Preterm Infants-no postnatal disease	C Healthy Term neonates
Number	27	32	27
Gestational age (range) Median gestational age (wks)	23 - 32 29	27 - 32 30	37 - 42 39
Weight (range) Median Birth Weight (grams)	690 - 2290 1050	790 - 2180 1555	2100 - 4400 3330
Length (cm, range) Median length (cm)	32 - 51 40	34 - 49 43	48 - 61 53
Male/Female Gender	17/10	16/16	15/12
Caesarean section (n/%)	20 (74%)	22 (69%)	11 (41%)
Natural labour and delivery	7	10	16
Apgar score at 1 minute after birth (range) Median Apgar Score	1 - 10 5	3 - 10 7	8 - 10 9
Apgar score at 5 minutes after birth (range) Median Apgar Score	3 - 10 6	3 - 10 8	9 - 10 10

Table 1: *Demographics of groups A, B and C.*

Ghrelin [pg/mL]	A Ill Preterm Infants	B Well Preterm Infants	C Healthy Term Infants	Statistical Significance
Mean ± SD	277 ± 337	327 ± 275	405 ± 337	$p = .15^{*}$
Median IQR (Q1 to Q3)	135 367 (39 - 405)	281 407 (74 - 481)	288 623 (86 - 706)	$p = .28^{\dagger}$

Table 2: *Concentrations of Ghrelin in Cord Blood in Groups A, B and C.*

**: Analysed with one-way ANOVA*

†:Analysed with Kruskal-Wallis Test

We performed multiple regressions to see if ghrelin levels were predictive of neonatal variables. Our analyses met the assumptions of linearity, independence of residuals (Durbin Watson = 2.4), homoscedasticity, collinearity (no correlations > .70, tolerance levels > 0.1), unusual points, and normality of residuals. As such, we did not perform a log transformation of the data.

We found that cord blood concentrations of ghrelin at birth significantly predicted stage I and II retinopathy of prematurity ($p < 0.02$, $R^2 = .37$).

Table 3 summarizes regression coefficients and standard errors.

Variable	Unstandardized Coefficients		Standardized Coefficients	p value	95% Confidence Interval for B	
	B	Std. Error	Beta		Lower Bound	Upper Bound
Gender	-61.03	96.910	-.102	.532	-256.5	134.4
Gestational Age	-52.2	44.741	-.385	.250	-142.5	38.1
Birth weight	-.089	.198	-.119	.672	-.485	.315
BPD	-138.3	179.3	-.188	.445	-499.8	223.3
Stage I - II ROP	250.1	105.6	.368	.022*	37.1	463.1
Stage III ROP Requiring Laser	-54.4	196.6	-.051	.783	-451.0	342.1
NEC	-138.4	191.2	-.118	.473	-524.1	247.3
Length	17.9	14.3	.299	.215	-10.8	46.7
Apgar at 1 minute	-29.8	31.4	-.253	.349	-93.1	33.6
Apgar at 5 minutes	47.8	44.0	.303	.284	-41.0	136.6
Days of Oxygen	1.7	6.0	.048	.780	-10.4	13.8
Days of CPAP	6.9	6.6	.248	.305	-6.5	20.2
Days of Ventilation	-7.8	7.8	-.276	.319	-23.5	7.9
IVH Grades 1 to 2	91.2	83.6	.152	.282	-77.7	260.1
IVH Grades 3 to 4	119.2	257.1	.088	.645	-399.5	637.8

Table 3: *Summary of Multiple Regression Analysis.*

**p < 0.05 is significant*

We also found ROP was associated with treatments administered for respiratory disease. As shown in table 4, there were statistically significant differences in rates of days of oxygen therapy, days of CPAP, and days of ventilation for infants with Stage I - II ROP as well as

Stage III ROP when compared to those without ROP. There was also a statistically significant relationship between ROP Stage III requiring laser ablation and grades 3 and 4 IVH ($p < 0.001$). Infants with IVH Grades 3 to 4 were 12 times more likely to require laser ablation (OR = 12.4, 95% CI = 3.2, 48.5).

Subjects	Disease	# of subjects (total # of subjects with disease)	Odds Ratio	95% CI	*p*-value
Females > 28 wks Ghrelin < 40 pg/mL	Stage I or II ROP	2 of 3 infants	2.0	0.55 - 7.31	0.55
Males > 28 wks Ghrelin < 40 pg/mL	Stage I or II ROP	2 of 4 infants	2.4	0.854 - 6.997	0.23
Females > 28 wks Ghrelin < 40 pg/mL	ROP stage III requiring laser ablation	1 of 1 infants	3.8	1.8 - 8.1	0.30
Group A Ghrelin < 40 pg/mL	Stages 3or 4 IVH	1 of 3 Infants	3.7	0.31 - 42.9	0.18

Table 4: *Odds Ratios and 95% Confidence Intervals in Infants with Ghrelin levels <40 pg/mL.*

We assessed measures of clinical significance such as odds ratio and found that infants > 28 wks gestation with ghrelin levels <40 pg/mL were more likely to have ROP Stage I - II, ROP Stage III, and IVH Grade 3 - 4 as shown in table 5. While the variables were not statistically significant, there is clinical significance based on the odds ratios and confidence intervals.

Finding	Groups A and B 59 total subjects	Mean Rank	Sum of Ranks	Z score	p value
IVH Grades 1 to 2†	34				
Days of Oxygen		32.44	1103.00	-2.281	0.06
Days of CPAP		32.62	1109.00	-1.380	0.16
Days of Mechanical Ventilation		34.19	1162.50	-2.280	0.02*
IVH Grades 3 to 4†	3				
Days of Oxygen		36.00	108.00	-.914	0.361
Days of CPAP		33.83	101.50	-.401	0.688
Days of Mechanical Ventilation		51.50	154.50	-2.321	0.02
ROP Stages 1 - 2†	15				
Days of Oxygen		35.93	539.00	-2.281	0.02*
Days of CPAP		38.40	576.00	-2.217	0.03*
Days of Mechanical Ventilation		35.57	533.50	-1.516	0.13
ROP Stage III Requiring Laser‡	**5 total subjects**				
IVH Grades 3 to 4	2				< 0.001*

Table 5: *Associations of Retinopathy of Prematurity with Respiratory Care and Grades 3 and 4 Intracranial Haemorrhage.*

**p < 0.05 is statistically significant*

†: Analyzed with a Mann-Whitney U test

‡: Analyzed by Chi-square test

Discussion

Figure 1 shows the actions of ghrelin and the physiologic pathways as a hormone in the foetus and new born infant. Thus, this study evaluated the association between cord blood concentrations of ghrelin and gestational duration, anthropometric measurements, and the occurrence of diseases after preterm birth. Previously, research identified a higher concentration of ghrelin in the cord blood and serum of female neonates [17]. An earlier study found no an association of umbilical blood ghrelin and gender [18] which agree sour finding in this report. Lower concentrations of ghrelin after caesarean rather than vaginal delivery have been described [18], but we did not find this association. Moreover, the concentration of ghrelin in cord blood did not differ significantly between sick preterm (Group A) and well preterm infants (Group B) born at ≤ 32 wks of gestation (Table 2). Many studies have looked for associations between birth weight and the concentration of ghrelin in maternal blood, cord blood, and neonatal serum. The majority of findings were negative with no correlation between the concentration of this hormone in cord blood and the birth weight of the neonate [19,20]. Studies report lower concentrations of ghrelin in cord blood of infants who had intrauterine growth restriction and a negative correlation with anthropometric measurements in a group of SGA infants [21]. The current study did not find such a relationship between ghrelin and fetal growth restriction. We suggest our population of healthy pregnant women in a country with a nation-wide health system resulted in fewer SGA infants.

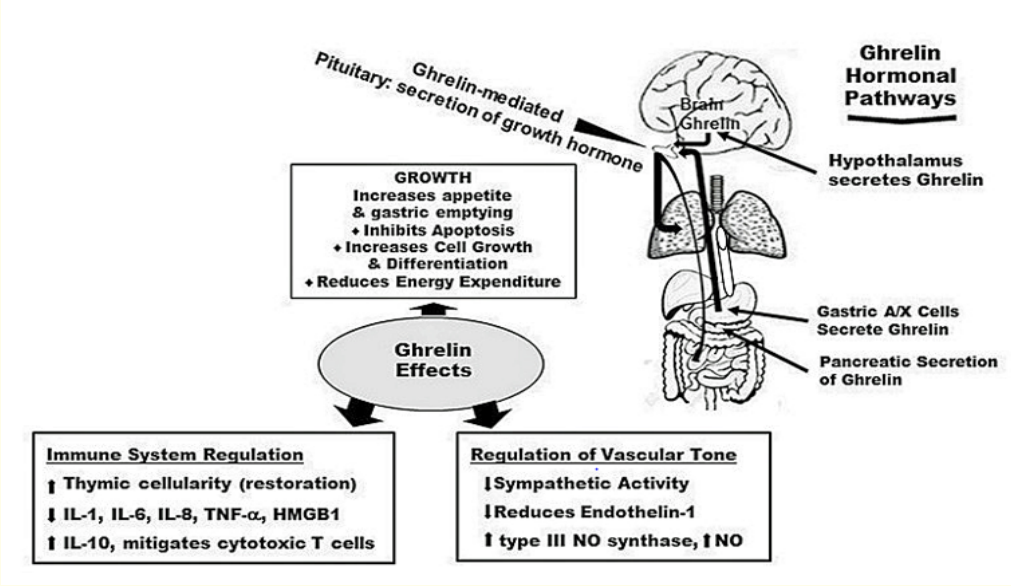

Figure 1: *Pathways and physiologic actions of ghrelin in the foetus and new-born infant. Linkage involves ghrelin-related blood or autocrine communication, but does not show ghrelin-related stimulatory effects via the vagal nerve. (Original Illustration created by Michael P. Sherman, MD).*

Since studies show the placenta produces ghrelin and thissecretagogue is present in amniotic fluid swallowed by the foetus, investigators in the future must examine the role of maternal uteroplacental diseases, like preeclampsia, and correlate placental weight and histopathology with the levels of ghrelin in cord blood. It is also crucial that researchers follow the kinetics of ghrelin production in blood after birth. This information may provide valuable information about the pathophysiology of ROP, BPD, and NEC.

Our strongest association was between umbilical blood ghrelin levels and ROP. A well-established concept is perinatal infection and inflammation [22] and angiogenesis [10] influence the presence and severity of ROP. Evidence shows ghrelin can mitigate inflammation

during infection and may have anti-bacterial activity [11,23-25]. Ghrelin may also alter vascular tone and blood flow, especially by down regulating endothelin-1 during sepsis [26]. A unique action of ghrelin is increased production of nitric oxide by endothelium [27] that likely enhances perfusion in the retinal microcirculation and thus minimizes the secretion of VEGF.

Infants born at > 28 wks post-conceptual age are at lower risk for developing ROP [28]; however, these infants in the current research study had a higher occurrence of stage I and II ROP if the cord blood concentrations of ghrelin was <40 pg/mL (Tables 3 and 4). Hence, low ghrelin levels in umbilical blood may identify infants at risk of ROP who caregivers usually consider lower risk. With a $R^2 = 0.37$, umbilical blood ghrelin concentration at birth was responsible for 37% of the effect on ROP. In association with care practices, Table 5 shows the association with ROP. Consistent with published reports [29], we found a strong relationship between grade grades 3 and 4 IVH and stage III ROP requiring laser ablation (Table 5).

Finally, the pregnant women in this study hinder extrapolation of the results to higher risk pregnant women and their preterm infants in other countries. The population of women in Poland are more homogeneous and are perhaps at lower risk. This study describes a small number of infants cared for at a single institution. Therefore, researchers must confirmed our findings in a larger and more diverse population at multiple perinatal care centres. We propose that adding other hormones, growth factors and biomarkers to ghrelin measurements in cord blood may help anticipate organ specific disease in preterm neonates. Importantly, future research in this field should perform serial measurements of ghrelin after birth while attempting to correlate the levels with ROP, inflammation and blood flow.

Conclusion

Previous studies of umbilical blood ghrelin have focused on the overall picture of fetal growth rather than this secretagogue's influence on specific organ growth and maturation and the relationship of cord blood concentrations of ghrelin at birth to diseases of prematurity. The number of preterm infants in this study is limited, and we anticipate larger populations at multiple sites may predict the occurrence of other organ specific diseases beyond that of ROP. Future research will identify if ghrelin is a quintessential hormone in fetal and neonatal growth, maturation, and protection.

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Conflict of Interest

There are no conflicts of interest or financial arrangements to disclose.

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