CLINICAL STUDY

Adiponectin levels measured in dried blood spot samples from neonates born small and appropriate for gestational age

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Abstract

Objective: Adiponectin levels measured in neonatal dried blood spot samples (DBSS) might be affected by both prematurity and being born small for gestational age (SGA). The aim of the study was to measure adiponectin levels in routinely collected neonatal DBSS taken on day 5 (range 3–12) postnatal from infants.

Design: A retrospective case–control study.

Subjects and methods: One hundred and twenty-two infants: 62 very premature (34 SGA) and 60 mature infants (27 SGA). Adiponectin concentrations were determined in stored neonatal DBSS using a sandwich immunoassay based on flow metric Luminex xMap technology.

Results: Adiponectin was measurable in all samples, and repeated measurements correlated significantly ($r=0.94$). Adiponectin concentrations were negatively associated with both SGA ($B=-0.283$, $P=0.04$) and prematurity ($B=-2.194$, $P<0.001$), independently of each other. In the premature but not the mature group, adiponectin levels increased with increasing postnatal age at blood sampling ($B=0.175$, $P<0.001$).

Conclusions: Reliable quantification of adiponectin in stored DBSS is feasible and may be used to study large populations of routinely collected samples. Low levels of adiponectin in neonatal DBSS are associated with SGA as well as prematurity. Blood adiponectin levels increase with postnatal age in premature infants, suggesting a rapid yet unexplained metabolic adaptation to premature extrauterine life.

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Introduction

During the first weeks of life, newborns face a rapid organ and metabolic adaptation to extrauterine life. Adiponectin may play an important role in this adaptation process due to its involvement in the regulation of insulin sensitivity, and thereby perhaps in early postnatal growth. In adults, adiponectin is involved in the regulation of insulin sensitivity (1, 2), and might predict future development of insulin resistance (3). High levels of adiponectin are associated with a reduced risk of developing type II diabetes (4), and adiponectin has an anti-atherogenic effect (5).

Previous studies on adiponectin levels in newborns were mainly performed using cord serum. Cord serum adiponectin concentrations were positively correlated to birth weight (BW) in most (6–10) but not all studies (11, 12), and some (6, 7, 10, 11) but not all (9) investigators found a positive correlation between gestational age (GA) and cord serum adiponectin levels.

Newborns had up to three times higher adiponectin levels than their mothers (9, 11), and in mature infants these high levels seemed stable during the first week of life (6, 8, 13).

Dried blood spot samples (DBSS), collected a few days after birth for newborn screening programs and then stored, are blood samples available for studies of pathological peri- and neonatal conditions. Since they are collected from populations with no selection and universal coverage, stored residual DBSS constitute a valuable resource for retrospective studies and epidemiological research. A newly developed multiplex sandwich immunoassay based on flow metric Luminex xMAP technology made it possible to measure multiple protein markers in the small amount of blood available in these samples (14). We have now used this technology to measure adiponectin in neonatal DBSS.

To our knowledge, only two partly contradictory studies have addressed adiponectin levels in small for gestational age (SGA) versus appropriate for gestational

age (AGA) newborns (12, 15). Therefore, we decided to investigate how SGA and prematurity affects adiponectin levels in newborns.

Subjects and methods

Study population

We included 122 infants in four groups: 1) premature SGA (*n* = 34); 2) premature AGA (*n* = 28); 3) mature SGA (*n* = 27); and 4) mature AGA (*n* = 33; for details see Table 1). Inclusion criterion for the premature groups was GA below 32 weeks and for the mature groups GA 37–42 weeks. SGA was defined as BW below –2 s.d. for GA, according to the intrauterine growth charts of Marsal *et al.* (16). The premature infants were admitted to our neonatal intensive care unit at Rigshospitalet in 2004, and only surviving infants were included. Mature SGA and AGA infants were identified at Statens Serum Institut (SSI) or Rigshospitalet. The infants were matched on birth date to diminish the problem of storage time of the DBSS across the groups, and the premature infants were further matched on GA to make sure these groups were comparable.

Samples

DBSS from the Danish neonatal screening program were used. According to the sampling instructions, capillary blood from the heel is spotted on filter paper (Schleicher & Schuell 903) preferably 5–8 days after birth, dried for 2–3 h, and mailed to SSI. After screening analysis, surplus DBSS are stored dry in a refrigerator for approximately a week before final storage at –24 °C in the biological specimen bank. To study the stability of adiponectin, blood from ten healthy adult volunteers was spotted on filter paper and left at room temperature

for 0, 3, 5, 7, 14, and 28 days respectively before storage at –24 °C.

Adiponectin assay

Adiponectin concentration in DBSS was measured with 14 other cytokines (interleukins 1β, 2, 4, 6, 8, 10, and 12, tumor necrosis factors α and β, neurotrophin-4, C-reactive protein, insulin-like growth factor-binding proteins 1 and 3, and leptin) by a multiplex sandwich immunoassay using Luminex xMAP technology essentially as described by Skogstrand *et al.* (2005) (14). Due to conflicts of interest and lack of validation of some of the proteins measured, only data on the adiponectin determinations are presented. In short, anti-human adiponectin (Cat. no. MAB1065, R&D Systems Inc., Minneapolis, MN, USA) was coupled to carboxylated beads (Luminex Corp., Austin, TX, USA) for use as capture antibody. Standard curves for adiponectin were made by spotting recombinant human adiponectin (Cat no. 1065-AP, R&D Systems) on filter paper models. Two filter paper disks, equivalent of 6 μl whole blood, were punched out and extracted in 180 μl buffer with protease inhibitor. The assay was subsequently setup for triplicate determinations. The 50 μl sample extract was incubated with a suspension of capture antibody-conjugated bead sets, including the anti-adiponectin-conjugated set, and the beads were washed as described (14). The captured antigens were reacted with a mixture of biotinylated detection antibodies, including biotinylated anti-human adiponectin (Cat. no. BAM1065, R&D Systems), and finally incubated with streptavidin–phycoerythrin. The beads were washed, re-suspended, and analyzed on the Luminex 100.

Standard curves were calculated by a five-parameter logistic equation (5PL), and goodness of fit expressed as standards recovery (back calculation of standards). Because of lack of sufficiently concentrated adiponectin, the standard curve was extrapolated by 5PL for readings of high sample concentrations. To evaluate the robustness of this extrapolation, determinations of adiponectin in DBSS from 56 infants in two different runs were compared. Determination of intra-assay coefficients of variance (CV%) was done by measuring eight identical samples in the same run and inter-assay CV% by measuring four identical samples in the same run repeated for 4 different days. Lower limit of detection (LOD) was defined as the concentration resulting in a response two s.d. away from zero dose response.

Anthropometrical data

BW, GA, date of blood sampling, and maternal age were obtained from hospital records or from SSI. The GA of some infants in the mature AGA group was given in full weeks, thus making these infants

Table 1 Characteristics of the study population.

	Premature		Mature	
	SGA group (<i>n</i> = 34)	AGA group (<i>n</i> = 28)	SGA group (<i>n</i> = 27)	AGA group (<i>n</i> = 33)
Adiponectin (μg/ml)*	6.2 ± 4.0	8.9 ± 6.1	12.8 ± 5.4	14.0 ± 3.9
Gestational age* (weeks)	29.0 ± 1.9	28.6 ± 2.0	39.9 ± 1.4	40.4 ± 1.1
Gender (M/F)	23/11	19/9	19/8	16/17
Birth weight* (g)	921 ± 252	1330 ± 348	2597 ± 212	3626 ± 431
Birth weight-SDS*	–3.05 ± 0.70	–0.28 ± 0.58	–2.29 ± 0.23	–0.05 ± 0.77
Postnatal age at blood sampling (days)*	5.9 ± 2.0 ^a	6.8 ± 2.2	6.2 ± 2.0 ^b	6.2 ± 1.9 ^c
Mother's age (years)	30.3 ± 4.5	31.0 ± 5.4	32.4 ± 5.2	29.7 ± 3.7

*Mean ± 1 s.d. ^a*n* = 33, ^b*n* = 26, ^c*n* = 32.

younger. To correct for this, the GA was calculated as full weeks plus three days. Three infants lacked date of blood sampling and were excluded from the analysis regarding this variable. In the premature groups, we obtained data on parity $n=53$ (27 SGA), mode of delivery (vaginal versus section) $n=55$ (28 SGA), and nutrition the day before blood sampling $n=39$ (20 SGA) from hospital records. Nutritional intake was calculated from 0600 to 0600 h the next day. The nutritional value of breast milk was set to 70 kcal/100 ml and the content of protein was set to 1.2 g/100 ml regardless of whether it was mother's milk or banked breast milk. The nutritional value of parenteral nutrition and fluids was obtained from the manufacturers.

Ethical considerations

The local ethics committee (KF (01) 259275 and KF (II) 271665) and the board of the biological specimen bank at SSI approved the study. The DBSS are stored at SSI in accordance with regulations from the Ministry of Health (17).

Statistical analysis

Square root transformation resulted in an approximate normal distribution, and was used in statistical analyses. A paired samples *t*-test was used to evaluate the correlation between paired data in the validation of the adiponectin assay. Differences between means were assessed by two-tailed *t*-test. Univariate ANOVA was used for individual variables and general linear models were constructed to test for multiple variables. SPSS statistical package version 13 was used (SPSS Inc., Chicago, IL, USA). A *P* value <0.05 was considered significant.

Results

The standard curve for adiponectin is shown as Fig. 1A. No cross reactivity to any of 14 other recombinant analytes was observed. The standards recovery was 92–144% (mean 113%) indicating a good fit of the curves. Intra- and inter-assay CV% was 5.9 and 12.2 respectively and the LOD was 0.0156 µg/ml. Correlation between adiponectin determinations in 56 neonatal DBSS obtained from two different runs was $r=0.94$ ($P<0.001$; Fig. 1B), and mean difference between the two runs was 14.6% which is of the same magnitude as the inter-assay CV%. The correlation between the two runs is lower at higher concentrations; however, we have very few samples in this area and therefore included them in our analysis. In a future study we might consider diluting the samples. Adiponectin concentrations declined steadily following

storage at room temperature, and this decline became significant after 5 days of storage at room temperature. The correlation between adiponectin concentrations at days 1 and 5 was excellent ($r=0.98$, $P=0.002$; Fig. 1C). Adiponectin in serum was 2.7 times higher than in DBSS (corrected for hematocrit) and determinations from the two media correlated well ($r=0.87$, $P=0.001$; Fig. 1D). Adiponectin levels in the mature AGA group were 2.5 times higher than in the adult group (data not shown).

The mean GA was 28.8 ± 1.9 weeks in the premature group and 40.2 ± 1.2 weeks in the mature group. Mean BW-SDS was -2.71 ± 0.66 and -0.15 ± 0.69 in the SGA and AGA groups respectively. The mean BW-SDS was lower in the premature than in the mature SGA group ($P<0.0001$), but did not differ between the AGA groups ($P=0.19$; Table 1). In univariate analysis, prematurity explained 31% ($r=0.55$, $P<0.001$) and SGA explained 6% ($r=0.24$, $P<0.008$) of the variation in adiponectin levels (Fig. 2A–C). Postnatal age at blood sampling was between 3 and 12 days and did not differ significantly between the four groups, hence the collection procedure for the neonatal DBSS was followed even in the two groups of very premature infants. Postnatal age at blood sampling explained 3.9% of the variation in adiponectin levels ($r=0.20$, $P=0.032$; Fig. 2D). If the data are extrapolated back to day 0, the adiponectin level in the mature group was 13.7 µg/ml when compared with 2.3 µg/ml in the premature group (untransformed data). Gender and maternal age did not explain any of the variation in adiponectin levels. In the premature group we had data on parity, mode of delivery, and nutritional intake. Neither parity nor mode of delivery explained any of the variation in adiponectin levels in this group. Mean nutritional intake the day before blood sampling was 86 ± 31 kcal/kg in the premature SGA group and 91 ± 21 kcal/kg in the premature AGA group ($P=0.57$). Kcal/kg explained 11% of the variation in adiponectin levels in the premature group ($r=0.34$, $P=0.035$). In a general linear model including BW-SDS, GA and kcal/kg as continuous variables, the association was weakened however ($P=0.06$).

A general linear model, including SGA versus AGA and premature versus mature as fixed factors, and postnatal age at blood sampling as a continuous variable, explained 43% of the variation in adiponectin levels. SGA was an independent predictor of adiponectin levels ($B=-0.283$, $P=0.04$) and so was prematurity ($B=-2.194$, $P<0.001$). There was a significant interaction between postnatal age at blood sampling and the grouping premature versus mature ($P=0.004$). In the premature but not the mature group, adiponectin levels were positively associated with an increase in postnatal age (Table 2). In an attempt to explain these findings, the co-variation between age at the day of blood sampling and kcal per kg body weight was tested

Figure 1 Adiponectin measurements in dried blood spot samples. (A) Standard curve for adiponectin in DBSS (F-adiponectin, concentration of adiponectin in dried blood spot samples). (B) Correlation between repeated measurements of adiponectin DBSS ($n=56$); the solid line is $x=y$; a Bland–Altman plot in square root transformed levels with mean difference ± 2 s.d. is inserted. (C) Influence of storage at room temperature on adiponectin concentrations in DBSS ($n=10$). (D) Correlation between adiponectin measured in serum when compared with DBSS.

in the premature group and we found a similar significant positive relation ($r=0.50$, $P=0.001$).

Discussion

Our results show that adiponectin can be reliably measured in stored filter paper (DBSS) and that neonatal levels are negatively associated with both SGA and prematurity, independently of each other.

Measurable adiponectin in DBSS decreased with storage time at room temperature. However, because the infants were matched on birth date, and the neonatal DBSS were collected and handled according to a strict national standardized procedure even in our neonatal intensive care unit, we do not suspect bias between our groups due to collection, mailing, or storage procedures. The correlation between adiponectin levels in DBSS and serum was satisfactory and the adiponectin levels in term AGA newborns was 2.5 times higher than the level found in healthy adults, which is of the same magnitude as found by other investigators (6, 18).

In our study, we found a strong negative effect of prematurity as others have done (6, 9, 10).

For the first time, the correlation between adiponectin levels and BW-SDS is studied in a cohort comprising both premature and mature infants divided in groups of AGA and SGA infants. Our results in the mature groups are in contrast to the findings by Kajantie *et al.* (12) who did not find a correlation between BW-SDS and adiponectin levels in their mature group; however, their span of BW-SDS was more narrow than ours. Our results are in accordance with the results of Kamoda *et al.* (15) finding a correlation between adiponectin levels and BW-SDS in premature infants. Therefore, we find it reasonable to suggest that low BW-SDS is associated with low adiponectin levels regardless of GA.

Adiponectin is an interesting cytokine due to its involvement in the regulation of insulin sensitivity in adults. However, the association between adiponectin levels and insulin sensitivity in infancy and childhood is intriguing. In adults, high adiponectin levels were associated with high insulin sensitivity (19). If this were the case in newborns, we would expect the

Figure 2 The relation of gestational age, birth weight, and postnatal age at blood sampling to adiponectin levels. (A–C) Variation of GA, BW, and BW-SDS within and among the four groups and their relation to the adiponectin levels. (D) Correlation between adiponectin levels and postnatal age at blood sampling. The solid and the dotted lines are the regression lines for the premature and the mature groups respectively. *Square-root transformed levels.

infants in the SGA and premature groups to be less insulin sensitive than the infants in the AGA and mature groups, who had higher adiponectin levels. In healthy term infants, other investigators found adiponectin levels negatively correlated with insulin levels when controlled for BW, BMI, or BW/BL ratio, which might indicate a positive correlation between adiponectin levels and insulin sensitivity present at birth (6). However, the literature on insulin sensitivity in newborns is conflicting. In a study by Gray *et al.* newborn SGA infants (day 1–65) showed higher insulin levels 1 h after an oral milk tolerance test than AGA infants (20). Contrasting this, a study on insulin sensitivity in premature neonates, within 14 days *post partum*, found no difference between SGA and AGA infants (21) and finally, term SGA newborns, studied 48 h *post partum*, were more insulin sensitive than their AGA peers as evaluated by an i.v. glucose tolerance test (22). This indicates

that research on insulin sensitivity and adiponectin levels in the newborn is still highly relevant. We only present data on adiponectin and find it interesting that adiponectin levels in newborns are positively correlated to body weight, contrasting with the results in adults, but they are in accordance with the expected low adiponectin levels in a group of infants who are at risk of developing insulin resistance later in life.

In accordance with other studies (6, 8), adiponectin levels were high shortly after birth in the mature groups. Interestingly, adiponectin levels increased rapidly with postnatal age in very premature infants. By days 11–12 *post partum*, the premature infants had adapted to adiponectin levels corresponding to those of mature infants, an increase that takes 10–12 weeks *in utero*. Kajantie *et al.* found cord adiponectin levels to be approximately six times higher in their mature infants when compared with their premature infants.

Table 2 Parameter estimates of square root-transformed adiponectin as dependent variable (n=119).

Variable	Category	B	95% CI	P value
Dependent variable: square-root adiponectin				
Intercept		3.782	3.132 to 4.433	<0.001
Gestational age	Premature group	−2.194	−3.074 to −1.313	<0.001
	Mature group	Baseline		
Birth weight-SDS	SGA group	−0.283	−0.555 to −0.012	0.041
	AGA group	Baseline		
Postnatal age at blood sampling	Premature group (1 day more)	0.175	0.085 to 0.265	<0.001
	Mature group (1 day more)	−0.007	−0.105 to 0.092	0.894

Extrapolating our data back to day 0, our findings are of the same magnitude. The study was too small to find out whether postnatal age, caloric intake or perhaps third factors were responsible for the change in adiponectin levels in premature infants. It is interesting that whereas maturation and preparation for extra-uterine life regarding adiponectin is probably gradual during the last trimester (12), apparently it is induced very rapidly after preterm birth. Further studies are needed to address this issue.

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