Influence of Growth During Infancy on Endothelium-Dependent Vasodilatation at the Age of 6 Months


Abstract—Low birth weight and accelerated infant growth are associated with cardiovascular disease in adulthood. Endothelial dysfunction is regarded as a precursor of atherosclerosis and is also related to infant growth. We aimed to examine whether an association between infant growth and endothelial function is already present during discrete periods of growth during the first 6 months of life in healthy term infants. A cohort of 104 newborns was studied in the first week after birth and reexamined at the age of 6 months. Maximum vasodilatation in response to acetylcholine (endothelium dependent) and nitroprusside (endothelium independent) was measured in the vasculature of the forearm skin, using laser Doppler flowmetry and iontophoresis. Growth was calculated as difference in Z scores for weight, length, weight-for-length, and head circumference. Multivariable multilevel linear regression was used for the analysis. Growth from 0 to 1 month (calculated as difference in weight) was the only window in the first 6 months of life that was significantly and inversely associated with endothelium-dependent vasodilatation at 6 months (b=−11.72 perfusion units per Z score, P=0.01 in multivariable analysis). Birth size was not important when considered simultaneously with infant growth. Maximum endothelium-independent vasodilatation was not associated with birth size or growth parameters. We conclude that growth in the first month of life is inversely associated with endothelium-dependent vasodilatation at the age of 6 months in healthy term infants, regardless of birth size. (Hypertension. 2012;60:1294-1300.) • Online Data Supplement

Key Words: endothelium • hypertension • pediatrics • developmental origins • postnatal growth

Birth weight is inversely associated with adult hypertension, cardiovascular disease, and other diseases.1,2 Accelerated postnatal growth (catch-up growth) is one of the proposed mechanisms to explain this relationship.3 More specifically, growth during infancy (mostly defined as 0–2 years) has been proven to be an important critical age window. Accelerated infant growth has been associated with adult cardiovascular risk factors, including reduced insulin sensitivity, an adverse lipid profile, and hypertension.4,5

Along with conventional risk factors for cardiovascular disease, preclinical, functional vascular impairment has also been linked to accelerated infant growth, including endothelial dysfunction.6 Endothelial dysfunction, considered a precursor of atherosclerosis in adults,7 is an important feature of hypertension and may even precede the development of hypertension.8 Furthermore, adolescent endothelial dysfunction is a sensitive early marker of an adverse cardiovascular phenotype.9

Although the associations between infant growth and adult adverse cardiovascular outcomes have been studied extensively in retrospect, studies prospectively investigating the early effects of infant growth are less common. In one study, small-for-gestational-age (SGA) infants with weight catch-up growth in the first year of life had higher levels of fasting insulin at the age of 1 year than SGA infants without catch-up growth or appropriate-for-gestational-age infants.10 In another study, a positive association was found between growth during the first 6 months of life and blood pressure at as early as the age of 3 years.11 With regard to endothelial function, however, there is a need for prospective studies with measurements early in life, because retrospective studies are more likely to be subject to bias (eg, recall bias).
In the present study, we collected follow-up data from a cohort of 104 newborns, in whom we have previously shown inverse associations between body size and endothelium-dependent vasodilatation. We performed these measurements in the forearm skin in the first week of life using laser Doppler flowmetry and iontophoresis of acetylcholine, an endothelium-dependent vasodilator. Data obtained by this technique are associated with markers of systemic endothelial function, are strongly correlated with flow-mediated dilatation of the brachial artery, and are linked to coronary microvascular function in adults.

The primary aim of the present study was to examine whether there is an association between (critical windows of) infant growth and endothelium-dependent vasodilatation already in the first 6 months of life in healthy term infants. Second, we examined whether body size at birth or early postnatal growth is more important in the development of endothelium-dependent vasodilatation in the first 6 months of life.

Methods
An extended Methods section is available in the online-only Data Supplement.

Subjects
As described previously in detail, from January 2006 to May 2010, healthy term newborns (gestational age 37–42 weeks) born at Maastricht University Medical Center who remained at the hospital for ≥3 days, could participate in the study.

Measurements
Endothelium-Dependent Vasodilatation
Measurements were performed in the first week of life and at the age of 6 months. Local microvascular skin blood flow was measured in perfusion units (PU) on the dorsal side of the forearm. These data were obtained by use of single-point laser Doppler flowmetry (Periflux System, Perimed AB, Sweden), before and after pharmacologically induced vasodilatation. Endothelium-dependent vasodilatation was induced by acetylcholine, and endothelium-independent vasodilatation was induced by nitroprusside. Administration of both drugs across the skin was realized by iontophoresis. The study protocol prescribed multiple successive doses resulting in a cumulative dose-response curve. For statistical analysis, the average perfusion values in a steady period of ≥30 seconds without significant movement artifacts were taken at baseline and at maximum attained perfusion.

Anthropometry
Birth weight was collected from the clinical records after it was measured on an electronic scale (accurate to 1 g) as part of the usual delivery protocol in our hospital. All other anthropometry was performed by 2 experienced researchers. All data points were converted into SD scores (Z scores), using reference data accurate to 1 day. Furthermore, we collected growth data (weight, length, and head circumference) from the infant and child welfare centers and extracted the measurements closest to 30 days (1 month) and 90 days (3 months).

Covariates
The following variables were collected from hospital records: sex, gestational age, maternal hypertension during pregnancy, gestational diabetes, and mode of delivery. Assisted pregnancy and parity were also considered potentially important, as was blood pressure of the infant. Blood pressure was measured on the arm using an automated blood pressure recorder shortly before or after assessment of vasodilatation, when the infant was quiet or asleep. Measurements were taken by an experienced researcher, with the newborn in the supine position or when held by one of the parents, using an arm circumference-adjusted cuff. Systolic, diastolic, and mean arterial blood pressures were recorded as mean of 2 measurements.

Parents were interviewed with regard to maternal smoking during pregnancy, breast feeding, parental ethnic background, level of education, and family history of cardiovascular disease. Level of education was expressed dichotomously, and family history of cardiovascular disease was analyzed in 4 groups. We always included the time point (either birth or 6 months) as a covariate in our analyses.

Statistical Analysis
To compare group means, the dependent t test was used. Multilevel linear regression with an unstructured covariance matrix describing the variance of the 2 time points was used to assess the associations among growth, covariates, and vasodilatation. All variables were tested separately in a basic analysis with maximum perfusion as the outcome variable and sex, time point, and baseline perfusion as covariates. Because we showed their importance previously, hypertension during pregnancy was included in the acetylcholine basic model, and gestational age was added to the nitroprusside basic model. Second, the variables with P<0.10 from the basic model were added to the basic birth size and growth models to create multivariable models. Third, the growth parameters that showed a P value <0.10 in the multivariable model when considered over the 0 to 6 months period were studied in more detail by decomposing growth into 3 age windows: 0 to 1, 1 to 3, and 3 to 6 months. These subwindows were tested separately and finally simultaneously to assess their relative importance. A 2 sided P value of <0.05 was considered significant, and a P value of <0.10 was considered a trend.

Results
Baseline Characteristics
From all 104 newborns included in the study, 90 attended the follow-up visit at the age of 6 months. We experienced technical problems during 3 of these 90 measurements, and 1 nitroprusside protocol could not be performed successfully. This yielded a total of 87 and 86 successful protocols of acetylcholine and nitroprusside, respectively. At birth, there were 102 and 94 successful measurements of acetylcholine and nitroprusside, respectively. The reason for the lower number of successful nitroprusside protocols at birth was that acquiring an acetylcholine measurement (always performed first) had sometimes taken too long for the newborn or the parents. The 90 infants who returned for a second visit did not differ from the 14 infants who were not measured for all birth, pregnancy, and general covariates, except for maternal educational level and maternal weight (both lower in the missing group). Maternal education level and weight were not related (P>0.90). To account for these differences, we additionally added maternal educational level and maternal weight as a covariate to all multivariable models, when these variables were not included already. In Table S1 in the online-only Data Supplement, the characteristics of all infants of whom vascular measurements were available from the second time point of our study (6 months) are shown. When the data from Table S1 were statistically tested, there was no difference in baseline perfusion between the acetylcholine and the nitroprusside protocols at birth and the age of 6 months (P=0.34 and P=0.55, respectively). At birth, maximum perfusion in response to acetylcholine was significantly lower than maximum perfusion in response to nitroprusside (69.8 PU versus 127.6 PU; P<0.0001); at the age of 6 months, this difference was no longer significant (117.5 versus 127.3 PU; P=0.06).
Table 1. Multilevel Linear Regression Coefficients (b) for Maximum Attained Perfusion

<table>
<thead>
<tr>
<th>Variables Tested</th>
<th>Acetylcholine</th>
<th>Nitroprusside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basic</td>
<td>Multivariable</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>b [95% CI]</td>
</tr>
<tr>
<td>Birth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (Z score)</td>
<td>104</td>
<td>5.51 [0.94, 10.09]</td>
</tr>
<tr>
<td>Length (Z score)</td>
<td>101</td>
<td>5.64 [0.07, 11.20]</td>
</tr>
<tr>
<td>Weight-for-height (Z score)</td>
<td>101</td>
<td>2.81 [-2.88, 8.49]</td>
</tr>
<tr>
<td>Head circumference (Z score)</td>
<td>104</td>
<td>8.46 [3.64, 13.27]</td>
</tr>
<tr>
<td>Growth*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (ΔZ score 0–6)</td>
<td>90</td>
<td>-7.49 [-14.20, -0.77]</td>
</tr>
<tr>
<td>Length (ΔZ score 0–6)</td>
<td>87</td>
<td>-4.58 [-11.64, 2.48]</td>
</tr>
<tr>
<td>Weight-for-length (ΔZ score 0–6)</td>
<td>87</td>
<td>-6.34 [-13.39, 0.72]</td>
</tr>
<tr>
<td>Head circumference (ΔZ score 0–6)</td>
<td>90</td>
<td>-2.03 [-9.50, 5.45]</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>104</td>
<td>-2.83 [-7.56, 1.90]</td>
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<tr>
<td>Hypertension during pregnancy (yes vs no)</td>
<td>x</td>
<td>x</td>
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<tr>
<td>Gestational diabetes (yes vs no)</td>
<td>104</td>
<td>-12.59 [-48.17, 23.00]</td>
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<tr>
<td>Smoking during pregnancy (yes vs no)</td>
<td>104</td>
<td>-4.97 [-21.04, 11.10]</td>
</tr>
<tr>
<td>Assisted pregnancy (yes vs no)</td>
<td>104</td>
<td>2.35 [-13.36, 19.05]</td>
</tr>
<tr>
<td>Delivery mode (cesarean section vs vaginal)</td>
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<td>10.25 [-3.20, 23.69]</td>
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<tr>
<td>Parity (multipara vs nullipara)</td>
<td>104</td>
<td>1.18 [-11.31, 13.68]</td>
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<tr>
<td>General</td>
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<td></td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>101</td>
<td>0.11 [-0.52, 0.73]</td>
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<tr>
<td>Ethnic background (non-Dutch vs Dutch)</td>
<td>104</td>
<td>-0.22 [-17.10, 16.67]</td>
</tr>
<tr>
<td>Breast feeding (yes vs no)</td>
<td>104</td>
<td>10.04 [-2.70, 22.79]</td>
</tr>
<tr>
<td>Education level father (low vs high)</td>
<td>104</td>
<td>-12.18 [-24.70, 0.35]</td>
</tr>
<tr>
<td>Education level mother (low vs high)</td>
<td>104</td>
<td>-2.62 [-15.05, 9.81]</td>
</tr>
<tr>
<td>Family history of cardiovascular disease (lowest vs highest risk)</td>
<td>104</td>
<td>9.84 [-11.90, 31.57]</td>
</tr>
<tr>
<td>Height of father, m</td>
<td>103</td>
<td>2.62 [-78.88, 84.12]</td>
</tr>
<tr>
<td>Weight of father, kg</td>
<td>102</td>
<td>-0.29 [-0.77, 0.19]</td>
</tr>
</tbody>
</table>

(Continued)
perfusion in response to acetylcholine (endothelium-dependent vasodilatation) was higher at the age of 6 months than at birth ($P<0.0001$), whereas maximum perfusion in response to nitroprusside (endothelium-independent vasodilatation) was similar at both ages ($P=0.89$). The exact age of measurement at birth did not influence vascular measurements in our sample, and exact age of measurement (in days) was not associated with maximum response to acetylcholine or nitroprusside in linear regression analysis ($P=0.83$ and $P=0.35$, respectively). Therefore, all measurements were regarded as measurements at either 0 or 6 months. In a total of 95 infants (91%), growth data from the infant and child welfare centers were retrieved. Within these 95 infants, the availability of growth data varied from 100% (weight at 3 months) to 83% (head circumference at 1 month).

### Acetylcholine (Endothelium-Dependent Vasodilatation)

Maximum skin perfusion in response to acetylcholine at the age of 6 months was positively associated with all measures of size at birth, except weight-for-length $Z$ score, in both the basic and the multivariable analyses (Table 1). However, none of these birth sizes, when included in the appropriate multivariable models for growth, remained significantly associated with maximum skin perfusion in response to acetylcholine at the age of 6 months: birth weight $b=0.62$, $P=0.85$; birth length $b=1.81$, $P=0.60$; birth weight-for-length $b=-0.64$, $P=0.87$; and head circumference at birth $b=5.96$, $P=0.08$. With regard to the growth parameters, growth expressed as change in weight-for-length was negatively associated with maximum perfusion ($b=-8.15$ PU per $Z$ score; $P=0.02$ in the multivariable analysis). There was a trend for an inverse association between growth in weight-for-length and maximum perfusion ($b=-6.95$; $P=0.06$).

When growth in weight and in weight relative to length was investigated in detail, we found that weight growth from 0 to 1 and from 1 to 3 months was associated with maximum perfusion after stimulation with acetylcholine, whereas weight growth from 3 to 6 months was not (Table 2). With regard to weight-for-length, only growth from 0 to 1 month showed a relationship with maximum perfusion. To determine which of the 3 subwindows of growth in weight and weight-for-length (0–1, 1–3, or 3–6 months) was most important, they were also tested simultaneously (Table 2). Weight growth from 0 to 1 month was the only subwindow significantly associated with maximum perfusion in response to acetylcholine ($b=-11.72$; $P=0.01$), and analogously weight-for-length growth from 0 to 1 month was solely associated with maximum perfusion ($b=-9.45$; $P=0.03$). Next, in a separate analysis, all SGA infants (birth weight <$p10$; $n=27$) were excluded from these final models. The results were similar, although nonsignificant for weight-for-length, despite a drop in number of participants in the model ($b=-14.72$; $P=0.009$ and $b=-10.40$; $P=0.052$ for weight and weight-for-length, respectively).

### Nitroprusside (Endothelium-Independent Vasodilatation)

Maximum skin perfusion in response to nitroprusside was not associated with any of the birth or growth parameters in the basic or the multivariable analysis (Table 1), it was, however, positively related to paternal education level (low versus high level; $b=-18.34$; $P=0.01$).

### Discussion

This prospective study showed that infant growth in weight was negatively associated with endothelium-dependent vasodilatation, already at the age of 6 months, independent of birth weight. More specifically, growth in weight (and weight-for-length) during the first month of life seemed to be of pivotal importance for the early development of endothelium-dependent vasodilatation.

The results from the present study are consistent with the finding that early postnatal growth (during the first 2 weeks of life) was associated with adolescent endothelial function in individuals born preterm. Moreover, we have shown a similar association at a much younger age and, notably, that the association is also present in healthy infants, born at term. In addition, we confirmed that the association between infant growth and endothelium-dependent vasodilatation is not confined to SGA infants but extends to appropriate-for-gestational-age infants. The latter emphasizes that even growth in the spectrum of a normal population may have negative effects on endothelium-dependent vasodilatation. It should also be noted that, in separate models, growth consistently showed a negative influence, in both an age period in which the infants

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**Table 1. Continued**

<table>
<thead>
<tr>
<th>Variables Tested</th>
<th>Acetylcholine</th>
<th></th>
<th></th>
<th></th>
<th>Nitroprusside</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basic</td>
<td>Multivariable</td>
<td></td>
<td></td>
<td>Basic</td>
<td>Multivariable</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$n$</td>
<td>$b$ [95% CI]</td>
<td>$P$</td>
<td>$n$</td>
<td>$b$ [95% CI]</td>
<td>$P$</td>
<td>$n$</td>
<td>$b$ [95% CI]</td>
</tr>
<tr>
<td>Height of mother, m</td>
<td>104</td>
<td>31.19 [−67.56, 129.94]</td>
<td>0.53</td>
<td>100</td>
<td>0.62 [-115.15, 116.40]</td>
<td>0.99</td>
<td>100</td>
<td>0.62 [-115.15, 116.40]</td>
</tr>
<tr>
<td>Weight of mother, kg</td>
<td>104</td>
<td>−0.37 [-0.80, 0.06]</td>
<td>0.09</td>
<td>100</td>
<td>0.11 [-0.39, 0.61]</td>
<td>0.66</td>
<td>100</td>
<td>0.11 [-0.39, 0.61]</td>
</tr>
</tbody>
</table>

Basic model acetylcholine was adjusted for baseline perfusion, time point, sex, and hypertension during pregnancy. Multivariable model acetylcholine included the basic model also adjusted for maternal education level, paternal education level, and maternal weight. Basic model nitroprusside was adjusted for baseline perfusion, time point, sex, and gestational age. Multivariable model nitroprusside included the basic model also adjusted for maternal education level, paternal education level, ethnic background, family history of cardiovascular disease, maternal weight, and paternal height.

*Growth parameters were always additionally adjusted for baseline value; so weight ($ΔZ$ score 0–6) was adjusted for weight $Z$ score at birth.

†There was a trend for a difference between the second lowest group (group 1) and the highest group; group 1 vs highest $b=−20.23$, $P=0.09$. 

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showed slow growth (0–1 month) and in a period in which they showed faster growth (1–3 months). A beneficial effect of breast feeding (which is associated with slower postnatal growth than formula feeding during the first months of life), as suggested by earlier work, could not be demonstrated in our study. This might be a consequence of our moderate sample size. Finally, our data underline the importance of an early critical growth window with that the endothelium is not able to adapt to the sudden rise in circulating blood volume accompanying fast growth and experiences permanent alterations in function. Finally, accelerated growth promotes adipocyte size in mice, which may increase the production of adipokines, which are detrimental to endothelial function, such as leptin and resistin, although adiponectin may have beneficial effects.

The present study describes the natural development of vascular function, namely a significant increase in endothelium-dependent vasodilatation from birth to 6 months and a steady endothelium-independent vasodilatation. With regard to the development of endothelial function, our data support a role for postnatal growth, rather than birth size, because the effects of birth size were diminished in the statistical models incorporating postnatal growth. The strong tendency of SGA infants to show accelerated postnatal growth has been proposed as an explanation for the identification of associations between birth weight and endothelial function in 9-year olds, adolescents, and adults. However, this poses a problem because SGA newborns already show signs of endothelial dysfunction at birth.

Similarly, slow intrauterine growth and fast postnatal growth may both be linked to impaired endothelial function later in life. It is possible that accelerated postnatal growth is a second hit, which adds to an already existing excess risk in SGA infants. We suggest that this second hit is more powerful than the effect of being SGA. This view is supported by our finding that birth weight was negatively associated with endothelium-dependent vasodilatation at 6 months when its effect was tested separately from growth but not in simultaneous analysis with growth. Because growth and birth weight are closely related, growth may override the effect of birth weight in statistical testing when its effect is more powerful. In line with our observations, the study by Singhal et al also showed a significant influence of birth weight on endothelial function at the age of 16 years, when growth was not included in the model. A multihit hypothesis for the development of cardiovascular and renal diseases has been proposed earlier, the first hit being low birth weight and the second hit being, for example, rapid weight gain.

Our study has several distinctive features in the field, the first being a prospective study that has taken many potential confounding factors into account. Second, our results can

Table 2. Multilevel Linear Regression Coefficients (b) for Maximum Attained Perfusion in Response to Acetylcholine

<table>
<thead>
<tr>
<th>Variables Tested</th>
<th>Tested Separately</th>
<th>Tested Simultaneously</th>
<th>Tested Simultaneously Without SGA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>b [95% CI]</td>
<td>P Value</td>
</tr>
<tr>
<td>Growth*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (∆Z score 0–1)</td>
<td>94</td>
<td>−11.97 [−19.79, −4.17]</td>
<td>0.003</td>
</tr>
<tr>
<td>Weight (∆Z score 1–3)</td>
<td>94</td>
<td>−12.67 [−22.82, −2.52]</td>
<td>0.02</td>
</tr>
<tr>
<td>Weight (∆Z score 3–6)</td>
<td>90</td>
<td>−5.83 [−19.01, 7.36]</td>
<td>0.38</td>
</tr>
<tr>
<td>Weight-for-length (∆Z score 0–1)</td>
<td>81</td>
<td>−8.39 [−16.40, −0.38]</td>
<td>0.04</td>
</tr>
<tr>
<td>Weight-for-length (∆Z score 1–3)</td>
<td>82</td>
<td>−4.41 [−13.27, 4.44]</td>
<td>0.32</td>
</tr>
<tr>
<td>Weight-for-length (∆Z score 3–6)</td>
<td>89</td>
<td>−4.30 [−13.22, 4.62]</td>
<td>0.34</td>
</tr>
</tbody>
</table>

*Change scores were always additionally adjusted for baseline value; so weight (∆Z score 0–6) was adjusted for weight Z score at birth.
be extrapolated to the general population to a high degree because of the healthy, term study population. However, our sample was recruited in an academic hospital and, for example, had a higher rate of cesarean sections than the general population. Third, we showed that the observed associations regarding infant growth were absent when an endothelium-independent vasodilator was used. This suggests that we found an exclusive association with endothelium-dependent vasodilatation and that this association was not influenced by the technique used. As an exception, we did observe an association between paternal educational level and endothelium-independent vasodilatation; however, because we do not have a plausible biological explanation for this, we expect it to reflect a type 1 error. Our study also has several limitations. As in any observational study, no conclusions can be drawn with regard to causality. Second, because of the modest study size, we were not able to explore potentially important interactions between growth and other variables. Third, we used laser Doppler flowmetry of the forearm skin to assess endothelial function because this is the most feasible technique for use in newborns and infants. Although endothelial dysfunction measured in the skin vasculature correlates well with cardiovascular disease risk in adults and endothelial dysfunction is thought to be a sensitive early marker in adolescence associated with an adverse later cardiovascular phenotype, we cannot be sure what the exact meaning of endothelium-dependent vasodilatation at this young age is and whether the observed associations will track into adulthood. Also, although peripheral vascular endothelial dysfunction is thought to play a role in hypertension and diabetes mellitus, at least in adult obesity, the association between our peripheral vascular measure and endothelial dysfunction in the conduit arteries (the main site of atherosclerosis) in infants is unknown. Finally, we have only studied infant growth, whereas it is clear that childhood growth may also have important effects on later cardiovascular health.

**Perspectives**

We have shown in a prospective study that growth in the first month of life is inversely associated with endothelium-dependent vasodilatation at the age of 6 months in healthy infants born at term, regardless of SGA or appropriate-for-gestational-age condition at birth. Birth size was not important when considered simultaneously with infant growth. These data underline the potential of counseling and intervening in early infant growth to prevent later cardiovascular disease and these data open up a new window (6 months to adolescence) in which a health benefit may be achieved with regard to endothelial function.

**Acknowledgments**

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**Disclosures**

None.

**References**

<table>
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<tr>
<th>Number</th>
<th>Author(s)</th>
<th>Title/Abstract</th>
<th>Journal</th>
<th>Year</th>
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</table>

**Novelty and Significance**

**What Is New?**
- This article describes the association between early infant growth and endothelial function based on a prospective study which followed term, healthy newborns until the age of 6 months.

**What Is Significant?**
- The first month of life is identified as a critical growth window in the development of endothelial function.

**Summary**
- Weight gain in the first 6 months of life, and especially in the first month of life, is inversely associated with endothelial function at the age of 6 months. These associations were independent of size at birth.
Influence of Growth During Infancy on Endothelium-Dependent Vasodilatation at the Age of 6 Months

Robbert N.H. Touwslager, Willem-Jan M. Gerver, Frans E.S. Tan, Marij Gielen, Maurice P. Zeegers, Luc J. Zimmermann, Alfons J.H.M. Houben, Carlos E. Blanco, Coen D.A. Stehouwer and Antonius L.M. Mulder

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THE INFLUENCE OF GROWTH DURING INFANCY ON ENDOTHELium-DEPENDENT VASODILATATION AT THE AGE OF SIX MONTHS

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Running title: Early growth and vascular function

Previous presentations of the present work: Parts of this work were previously presented as a poster presentation at the PAS/ASPR Conference in Denver, 2011 and as an oral presentation at the International Conference on Nutrition and Growth in Paris, 2012.

Contents: extended methods and supplemental table S1
Extended Methods

Subjects
As described previously in detail\(^1\), from January 2006 to May 2010, healthy term newborns (gestational age 37-42 weeks) born at Maastricht University Medical Centre, The Netherlands, who remained at the hospital for at least three days could participate in the study. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre and all procedures followed were in accordance with local guidelines. All parents gave written informed consent.

Measurements

Endothelium-dependent vasodilatation
The methods of measuring endothelium-dependent vasodilatation in our study were reported before in detail\(^1\). In short, measurements were performed in the first week of life and at the age of six months. At birth, measurements were taken shortly after feeding, when the children were asleep. At the age of six months, the infants were measured lying in their own baby seat, mostly awake. The forearm was gently fixated during measurements to minimize movement artifacts. Local microvascular skin blood flow of the dorsal side of the forearm was measured using single-point laser-Doppler flowmetry (Periflux system, Perimed A.B., Sweden) before and after pharmacologically induced vasodilatation. The probe temperature was fixed to \(32^\circ\) Celsius. Blood perfusion was recorded in perfusion units (PU), which correlate with the number and velocity of passing blood cells in the microvasculature of the skin. Endothelium-dependent vasodilatation was induced by acetylcholine, which is known to release NO and prostanoids from the endothelium, with a possible accessory role for endothelium-derived hyperpolarization factor in adults\(^2\). Endothelium-independent vasodilatation was induced by nitroprusside, a direct NO donor\(^3\). Administration of both drugs across the skin was realized by iontophoresis. The study protocol prescribed multiple successive doses resulting in a cumulative dose response curve. For acetylcholine (1% solution, Miochol, Novartis Pharma) 7 successive doses were administered with a 60 second interval, and for nitroprusside (0.1% solution, sodium nitroprusside) 9 doses with a 90 second interval were used. Administration required 20 seconds of iontophoresis using a 0.10 mA anodal current (acetylcholine) or a 0.20 mA cathodal current (nitroprusside)\(^4\). For statistical analysis, the average perfusion values in a steady period of at least 30 seconds without significant movement artifacts were taken at baseline and at maximum attained perfusion by use of specially designed software (Perisoft, Perimed A.B., Sweden). All analyses of the Laser-Doppler signal were conducted by one experienced researcher (RNT).

Anthropometry
Birth weight was collected from the clinical records, after it was measured on an electronic scale (accurate to one gram) as part of the usual delivery protocol in our hospital. All other anthropometry was performed by two experienced researchers. Supine length was measured using a measuring table accurate to 1 millimeter. Head circumference was measured using a non-stretchable tape, accurate to 1 millimeter, around the most protruding points of occiput and forehead. All standardizations into standard deviation scores were performed accurate to one day. For birth weight, standard deviation scores (z-scores) were calculated using sex, parity and gestational age adjusted reference data from ‘The Netherlands Perinatal Registry’\(^5\). The Dutch growth data published in ‘Paediatric Morphometrics’\(^6\) were used for the standardization of other measures of body size at birth. At the age of six months, z-scores were calculated using specially designed software from the Dutch Growth Foundation (Growth Analyzer, version 3), according to the Dutch reference data from 1997\(^7\). These reference data did not include gestational age adjusted data at birth and were therefore not used to standardize anthropometric measures at birth. They also did not include weight-for-
length references, so the data from ‘Paediatric Morphometrics’ were also used to calculate weight-for-length z-scores at the age of six months.

Furthermore, we collected growth data (weight, length and head circumference) from the infant and child welfare centers and extracted the measurements closest to 30 days (1 month) and 90 days (3 months).

Covariates
The methods of collection and scaling of covariates were described in detail before. The following variables, the selection of which was based on the literature, were collected from hospital records: sex, gestational age (based on the first day of the last menstrual period, recorded accurate to one day), maternal hypertension during pregnancy (includes preexistent hypertension, gestational hypertension, preeclampsia and HELLP syndrome), gestational diabetes and mode of delivery. Assisted pregnancy (including hormonal stimulation of ovulation, in vitro fertilization and intracytoplasmic sperm injection) and parity were also considered potentially important, as was blood pressure of the infant. Blood pressure was measured on the arm using an automated blood pressure recorder (at birth: Philips M1008B module, Eindhoven, The Netherlands; at six months: Dinamap Pro 300, GE Healthcare, Chalfont St. Giles, United Kingdom) shortly before or after assessment of vasodilatation, when the infant was quiet or asleep. Measurements were taken by an experienced researcher, with the newborn in the supine position or when held by one of the parents, using an arm circumference-adjusted cuff. Systolic, diastolic and mean arterial blood pressures were recorded as mean of 2 measurements.

Parents were interviewed with regard to maternal smoking during pregnancy, breast feeding, parental ethnic background (Dutch descent or not), level of education and family history of cardiovascular disease. Level of education was expressed dichotomously: 0—from no education to lower secondary education, 1—from intermediate level secondary school to tertiary education. Family history of cardiovascular disease was analyzed in four groups: 0= no known cardiovascular diseases in the family, 1=hypertension, hypercholesterolemia or type 2 diabetes in a second-degree relative <60 years, 2=cardiovascular event (transient ischemic attack, stroke or myocardial infarction) in a second-degree relative <60 years and 3=hypertension, hypercholesterolemia, type 2 diabetes or a cardiovascular event in a first-degree relative. Only one infant had a cardiovascular event in a first-degree relative. Since vascular function possibly changes during the first six months of life, we always included the time point (either birth or 6 months) as a covariate in our analyses.

Statistical analysis
Inter-observer variability for the analysis of the obtained signal in a random subsample of 20 newborns was assessed before and reached an intra-class coefficient of >0.98 for all perfusion values. To compare group means, the dependent t-test was used. Multilevel linear regression with an unstructured covariance matrix describing the covariance between the two time points was used to assess the associations between growth, covariates and vasodilatation. A two-level model takes into account that the two measurements in the study were performed in the same infants. Multilevel modeling uses the data of all infants, even if their outcome values are missing at a certain time point. First, all variables were tested separately in a basic analysis with maximum perfusion as the outcome variable and sex, time point and baseline perfusion (to account for baseline perfusion differences) as standard covariates. Since we showed their importance previously, hypertension during pregnancy was included in the acetylcholine basic model and gestational age was added to the nitroprusside basic model. Second, the variables with $P<0.10$ from the basic model were added to the basic birth size and growth models to create multivariable models using the enter approach. The $P$-value of 0.10 was chosen in order not to falsely ignore any potentially important covariates. Third, the growth parameters that showed a $P$-value <0.10 in the multivariable model when considered over the zero to six
months period, were studied in more detail by decomposing growth into three age windows: 0-1, 1-3 and 3-6 months. These sub-windows were tested separately and finally simultaneously to assess their relative importance. A two sided P-value of <0.05 was considered significant, a P-value of <0.10 was considered a trend. All analyses were performed using PASW Statistics version 18.0.

References


Supplemental Table S1. Infant Characteristics (n=87)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean/ n</th>
<th>SD/ %</th>
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**Birth**
- Age at Day of Measurement (days; mean, SD) 2.1 1.2
- Weight [Z-Score; mean, SD] -0.33 1.3
- Baseline Perfusion Acetylcholine Protocol (PU; mean, SD) 17.2 9.4
- Maximum Perfusion Acetylcholine Protocol (PU; mean, SD) 69.8 43.0
- Baseline Perfusion Nitroprusside Protocol (PU; mean, SD) 18.6 10.2
- Maximum Perfusion Nitroprusside Protocol (PU; mean, SD) 127.6 59.8

**6 Months**
- Age at follow-up visit (days; mean, SD) 196 20
- Weight [Z-Score; mean, SD] -0.08 1.0
- Baseline Perfusion Acetylcholine Protocol (PU; mean, SD) 15.9 12.1
- Maximum Perfusion Acetylcholine Protocol (PU; mean, SD) 117.5 49.4
- Baseline Perfusion Nitroprusside Protocol (PU; mean, SD) 14.6 12.4
- Maximum Perfusion Nitroprusside Protocol (PU; mean, SD) 127.3 46.1

**Growth**
- Age at One Month Measurement (days; mean, SD) 30 4
- Age at Three Months Measurement (days; mean, SD) 89 7
- Weight [Δ Z-Score 0-6; mean, SD] 0.25 1.21
- Weight [Δ Z-Score 0-1; mean, SD] -0.28 0.79
- Weight [Δ Z-Score 1-3; mean, SD] 0.53 0.74
- Weight [Δ Z-Score 3-6; mean, SD] -0.01 0.55

**Pregnancy**
- Gestational Age [week; mean, SD] 39.3 1.3
- Hypertension During Pregnancy [n, %] 17 19.5
- Gestational Diabetes [n, %] 4 4.6
- Smoking during Pregnancy [n, %] 16 18.4
- Assisted Pregnancy [n, %] 15 17.2
- Caesarean section [n, %] 63 72.4
- Multipara [n, %] 34 39.1

**General**
- Male [n, %] 36 41.4
- Systolic Blood Pressure at Six Months [mmHg; mean, SD] 89.3 11.5
- Non-Dutch Ethnic Background [n, %] 13 14.9
- Breast Feeding [n, %] 59 67.8
- Low Education Level Father [n, %] 29 33.3
- Low Education Level Mother [n, %] 32 36.8
- Family History of Cardiovascular Disease [n, %]
  - No Known Cardiovascular Disease 24 27.6
  - Risk Factors in Second Degree Relative 28 32.2
  - Event in Second Degree Relative 24 27.6
  - Risk Factors or Event in First Degree Relative 11 12.6
- Height Father [m; mean, SD] 1.81 0.08
- Weight Father [kg; mean, SD] 82.1 13.4
- Height Mother [m; mean, SD] 1.69 0.06
- Weight Mother [kg; mean, SD] 68.5 14.3