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Inflammation, lipids and aortic intima-media thickness in newborns following chorioamnionitis

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Short title: Inflammation in newborns after chorioamnionitis

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Abstract

Aim: This study investigated whether chorioamnionitis was associated with increased inflammation, dyslipidaemia, and adverse cardiovascular phenotypes in the immediate postnatal period. **Methods:** This prospective case-control study included preterm infants (30^{+0} - 35^{+6} weeks gestational age, GA) whose mothers did not have pregnancy-related conditions that may influence outcomes. Chorioamnionitis was diagnosed by placental histology and infants were divided retrospectively into cases (chorioamnionitis-exposed) and controls (unexposed). Serum high sensitivity C-reactive protein (hsCRP), lipid profile, far-wall abdominal aortic intima-media thickness (aIMT) and blood pressure (BP) were measured in the first week of life. **Results:** There were 20 (16 male, mean GA 32.4 weeks) cases and 31 (12 male, mean GA 32.6 weeks) controls. Histological chorioamnionitis was associated with a significant increase in hsCRP, and a non-significant trend towards an adverse lipid profile. There was no evidence of differences in aIMT or BP. **Conclusion:** Preterm infants exposed to chorioamnionitis have greater postnatal inflammation. There were no early postnatal differences in aIMT or BP. The inflammatory stimulus of chorioamnionitis late in gestation may be of insufficient intensity and duration to result in immediate postnatal alterations to arterial structure. Cardiovascular follow-up of infants exposed to chorioamnionitis may identify differential risk trajectories and subsequent inflammatory responses.

Keywords

Atherosclerosis, Chorioamnionitis, Neonatal, Perinatal health, Pregnancy

Key notes

- *In utero* factors during fetal development influence early postnatal intermediate cardiovascular phenotypes in offspring, but the role of perinatal inflammation (chorioamnionitis) is unknown.
- We report that preterm infants exposed to chorioamnionitis have increased postnatal inflammation, but no immediate postnatal differences in aortic intima-media thickness or blood pressure.
- Cardiovascular follow-up of infants exposed to chorioamnionitis may identify differential risk trajectories and subsequent inflammatory responses

Introduction

Atherosclerosis is a chronic inflammatory process beginning in early life, which may initiate *in utero*. Initial accumulation of immune cells and lipid within the arterial intima-media causes smooth muscle proliferation and further recruitment of inflammatory cells (1). Autopsy data show that the fetal distal aorta is the primary site of lesion formation (2). Approximately half of infants <6 months have coronary artery lesions (3). Although atherosclerotic lesions may regress during early childhood, the frequency appears to increase again in late childhood and adolescence (3), particularly in those exposed to risk factors that are highly predictive of atherosclerosis progression in later life (4). Although atherosclerosis is universal by adulthood, early life factors may influence the considerable inter-individual variation in severity.

The initiation and progression of atherosclerosis is associated in part with exposure to perinatal factors, including abnormal fetal growth and birth size, maternal diabetes, dyslipidaemia, and smoking (5). All of these factors are associated with an increase in newborn aortic intima-media thickness (aIMT), a marker of pre-clinical atherosclerosis (6). Aortic IMT is assessed by ultrasound and is a reliable and reproducible tool in newborn infants. It is recommended by the American Heart Association as part of the non-invasive assessment of pre-clinical atherosclerosis in children and adolescents (7).

In newborn infants with increased aIMT, inflammatory mediators are significantly increased in amniotic fluid (8), and in maternal (9), cord (10), and childhood plasma (11). High sensitivity C-reactive protein (hsCRP) is a widely used, dynamic and sensitive marker of inflammation, though its functional significance in atherosclerosis is unclear (12). A pro-atherogenic lipid profile (elevated total cholesterol, low density lipoprotein (LDL) and triglycerides) is associated with increased newborn aIMT and is a well-established risk factor for atherosclerosis in adulthood (13). These data are consistent with the consensus that chronic inflammation and dyslipidaemia are central to atherosclerosis onset and progression (14). Despite this, there are no data on the relationship between perinatal inflammation and the development of subclinical atherosclerosis in newborn infants.

Perinatal inflammation (chorioamnionitis) of the placental chorionic plate and fetal membranes is associated with ascending infection that triggers a maternal inflammatory response. In severe cases, chorioamnionitis results in a fetal inflammatory response involving the umbilical vessels (which originate from the abdominal aorta) and Wharton's jelly (funisitis) (15). Maternal symptoms are poorly predictive of chorioamnionitis and consequently, semi-quantitative histological staging assessment of inflammation of the placenta, membranes and cord is the diagnostic standard. Chorioamnionitis affects up to one in five term, and a third of preterm pregnancies (16). It is associated with a range of adverse outcomes in ex-preterm infants including retinopathy of prematurity, cerebral white matter damage, and pulmonary and systemic hypertension. Furthermore, the effects of chorioamnionitis on postnatal immune function alter the risk of newborn and childhood infections, such as late-onset neonatal sepsis and respiratory infections (15). Animal models suggest that lambs exposed to intrauterine inflammation develop persistently increased pulmonary and systemic arterial pressures (17). There are limited human data on the cardiovascular sequelae of chorioamnionitis.

The aim of this study was to investigate the association between histological chorioamnionitis and markers of pre-clinical atherosclerosis (aIMT, BP and serum biomarkers) in preterm infants exposed to chorioamnionitis and gestational age (GA)-matched controls.

Patients and methods

Study population

This prospective case-control study was carried out at the Royal Women's Hospital (RWH), Melbourne between August 2013 and June 2014 following institutional ethics approval. All newborn infants admitted to the intensive and special care unit between 30^{+0} and 35^{+6} weeks GA were assessed for eligibility. We selected moderately preterm infants as potentially confounding co-morbidities are less common and chronic (as opposed to acute) chorioamnionitis is more common than in extremely preterm births. Exclusion criteria were maternal conditions associated with abnormal vascular parameters in the offspring, including maternal renal impairment, pre-existing or gestational diabetes mellitus, pre-eclampsia, autoimmune, autoinflammatory, oncological or other conditions treated with immune-modulating drugs during pregnancy. Infants with major congenital abnormalities, aneuploidy or intrauterine growth restriction (<10^{th} centile) were excluded. Written informed consent was obtained from the parent(s) of all eligible infants within 72-hours postpartum.

Clinical assessment

On day three of life 1 mL of blood was collected in conjunction with clinical tests from each infant by heel prick or venepuncture. Serum was separated by centrifugation, snap-frozen and stored at -80°C for batch analysis. Serum samples were analysed for hsCRP, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides. Within 7 days postpartum the abdominal aortic artery was imaged by trans-abdominal ultrasound using a high-frequency General Electric (GE) 12L-RS linear array transducer (5-13MHz). At least three independent high-resolution images of a straight, non-branching 1cm segment of the proximal abdominal aorta of each infant were obtained. Images were captured at the end of diastole (gated on the ECG 'R' wave) and a 10mm segment of the far wall was analysed using CarotidAnalyzer automated offline edge-detection software (version 6, Medical Imaging Applications). Abdominal aIMT was measured as the distance from the lumen-intima interface to the media-adventitia interface. Maximum, minimum and

mean abdominal aIMT, as well as the minimum aortic lumen diameter, were recorded for each image and averaged across all images for each newborn infant. Three individual pre-ductal (right arm) blood pressure measurements were obtained for each infant using a standard neonatal Dinamap BP cuff appropriate for infant size, and the arithmetic mean used for analysis. All clinical assessments were completed blinded to placental histology.

Placental pathology

Samples of placenta, umbilical cord and chorioamniotic membranes were collected at all births <36 weeks gestation at the RWH and examined for evidence of inflammation. Using standardised techniques, tissues were formalin-fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin for microscopic investigation. The presence and extent of neutrophil polymorph infiltration in each tissue type determined the severity of both the maternal and fetal inflammatory response. The maternal inflammatory response is characterised by neutrophil accumulation in the subchorionic fibrin, chorionic plate, membranous connective tissue and/or amnion, and in severe cases necrosis. Neutrophil migration into the chorionic plate and umbilical vessels and Wharton's jelly represents a fetal inflammatory response.

Newborns were categorized retrospectively into cases and controls by placental histopathology. Cases had histological evidence of inflammation, whereas controls had no inflammation. Cases were also subdivided into "maternal" (evidence of maternal inflammatory response only) and "fetal" (evidence of maternal and fetal inflammatory response) to allow investigation of chorioamnionitis severity.

Statistical Analysis

Analysis was performed using Stata/IC version 13.0 (StataCorp LP, Texas, USA). Percentages were calculated for categorical variables, whereas means and standard deviations were determined for continuous variables. Fisher's exact test and Student's t-test were used to compare categorical and continuous variables between groups (cases and controls), respectively. Welch's t-test was used to compare

continuous variables in cases of unequal variance. Univariate analysis investigating the association between the presence / absence of chorioamnionitis and the outcome variables (aIMT and BP) was done using a single model linear regression. Single model multivariate regression was also conducted to adjust for birth weight z-score, preterm premature rupture of membranes (PPROM), infant sex, and minimum aortic lumen diameter (to account for differences in aIMT that occur with differences in growth). We analysed the following parameters in relation to aIMT; PPROM, maternal antibiotic use during pregnancy or labour, GA, birth weight, birth weight z-score, infant sex, BP, Apgar scores at 1 and 5 minutes, newborn anthropometry and serum biochemistry measurements, using single model linear regression. Outcome variables (aIMT, BP and serum biochemistry) were assessed based on the severity of chorioamnionitis using one-way analysis of variance (ANOVA) followed by a *post hoc* Scheffe test in cases of statistical significance. In all tests, $P<0.05$ was considered statistically significant.

Results

Study population

A total of 51 preterm newborn infants were recruited and assessed. Placental pathology confirmed chorioamnionitis in 20 infants (6 with maternal inflammatory response and 14 with maternal and fetal inflammatory response) and 31 infants were controls. Characteristics of the study population are shown in Table 1.

Chorioamnionitis was associated with a significant increase in antibiotic exposure during pregnancy (85% vs. 35%, $P<0.01$), an obstetric diagnosis of chorioamnionitis (50% vs. 6%, $P<0.01$) and the presence of pathogenic vaginal organisms (75% vs. 29%, $P<0.01$). The mode of delivery was similar between groups, except for an increased proportion of Ventouse forceps deliveries in the chorioamnionitis group (5% vs. 3%, $P=0.03$). Males were significantly over-represented in the chorioamnionitis group (80% vs. 39%, $P=0.01$); all other infant characteristics were similar between groups.

Serum Biochemistry

Biochemical data were available from 19 infants with chorioamnionitis (5 with maternal inflammatory response only and 14 with both maternal and fetal inflammatory response) and 29 controls. Chorioamnionitis was associated with a significant increase in hsCRP (mean CRP 2.93 vs 0.45mg/L; *P*=0.01; Table 2), which was most marked between cases with maternal inflammatory response and controls (P<0.01; Table 3). Other biochemical measurements were not significantly different between the groups, although there was a trend towards a pro-atherogenic lipid profile in cases (Table 2).

Cardiovascular measurements

There was no significant difference in aIMT (mean 0.35 vs. 0.36 mm, maximum 0.48 vs. 0.50 mm and minimum 0.41 vs. 0.42 mm) or BP (systolic 69.95 vs. 68.74 mmHg, diastolic 46.95 vs. 42.29 mmHg and mean 52.79 vs. 51.52 mmHg,) in cases versus controls (respectively) (Tables 3 and 4). There was also no significant relationship between aIMT and BP, serum biochemistry measurements, or clinical data.

Discussion

Moderately preterm infants born following pregnancies complicated by histological chorioamnionitis had evidence of persisting systemic inflammation at 72 hours of age, but no differences in aIMT or BP compared to controls. There was a non-significant trend towards a pro-atherogenic lipid profile in chorioamnionitis-exposed infants. Increased umbilical cord blood CRP has been reported in infants exposed to chorioamnionitis and those mounting the fetal inflammatory response *in utero* (18). Our results build on these findings by showing that inflammation continues after birth and persists into the early postnatal period. In adults hsCRP is a widely-used marker for cardiovascular risk assessment and is associated with atherosclerosis and altered blood pressure (19). Reduced mean and diastolic BP (20) and increased left ventricular compliance (21) have been reported in infants exposed to chorioamnionitis.

There was some evidence that infants exposed to chorioamnionitis had higher total cholesterol, LDL and triglycerides, and lower HDL than controls, indicative of a pro-atherogenic lipid profile. Similar lipid alterations and increased aIMT have been reported in infants born to mothers with preeclampsia (13) and macrosomic infants exposed to diabetes *in utero* (22). The prolonged *in utero* exposure in these conditions may result in vascular remodelling, reflected in postnatal changes in aIMT. In contrast, the relatively brief inflammatory stimulus from chorioamnionitis late in gestation may be insufficient to affect postnatal arterial structure, or these changes may occur later in infancy. Serial aortic ultrasounds may be warranted to track arterial remodelling following chorioamnionitis.

Emerging evidence suggests that the innate immune system may be primed upon first exposure to a stimulus and subsequently elicit a heightened inflammatory response to future insults (23). Previous studies have shown that chorioamnionitis is associated with a decrease in late-onset sepsis and respiratory distress syndrome (RDS), presumably due to induced maturation and the stimulatory effects of the fetal immune system *in utero*. Conversely, chorioamnionitis has also been linked with amplified pulmonary inflammation, surfactant dysfunction, increased chronic lung disease, cerebral palsy and early-onset sepsis (15). Infants exposed to chorioamnionitis may be prone to a heightened inflammatory response to subsequent postnatal stimuli, including childhood infection (to which they have heightened overall susceptibility (24)), and other pro-atherogenic determinants, such as dyslipidaemia, metabolic syndrome, and cigarette smoke. We have recently reported that childhood infection is associated with increased adult cardiovascular disease, and abnormal risk parameters, including obesity (25). Longitudinal investigation of infants exposed to chorioamnionitis is warranted.

Our methodological approach has a number of strengths. Firstly, we used abdominal aIMT as a marker of pre-clinical atherosclerosis, which may be a more discriminatory measure of subclinical atherosclerosis than carotid IMT (cIMT), and is easier to image in infants. Aortic IMT shows greater overall thickness, more inter-individual variability, and greater increase with age (26). The abdominal aorta is the first site of atherosclerosis development (2). Unlike many studies, we adjusted aIMT measurements for vessel size, as unadjusted measurements may result in spurious

associations. We also used semi-automated IMT image analysis software, which is more reproducible than caliper measurement of IMT (27). Secondly, the incidence of histological chorioamnionitis is inversely related to GA at birth (28), but it becomes increasingly difficult to assess the independent effects of chorioamnionitis as gestation decreases and neonatal morbidity and mortality increase. We therefore focussed on moderately preterm infants and applied strict exclusion criteria in an attempt to identify the effects of chorioamnionitis independent of complications associated with prematurity. Thirdly, retrospective allocation of participants to study groups enabled blinding of investigators during recruitment and clinical assessment. Finally, collection of newborn blood samples on day 3 of life more accurately reflects the infant's lipid profile, which may be altered in cord blood by maternal and perinatal factors (29).

We acknowledge some limitations, particularly the modest sample size. Previous analogous studies of abdominal aIMT in high-risk infants, such as those with growth restriction, have used group sample sizes of 25 infants (30) and demonstrated significant differences between these infants and controls. However, despite recruiting a similar number of participants we found no significant effects of chorioamnionitis on abdominal aIMT. Furthermore, the number of chorioamnionitis cases with only the maternal inflammatory response was limited, restricting our findings related to chorioamnionitis severity. Notwithstanding, our data do not suggest that increasing the overall sample size would alter the findings on aIMT or BP, although more participants may clarify the trends observed in lipid profile.

In conclusion, chorioamnionitis was not associated with increased abdominal aIMT and BP. Infants exposed to inflammation *in utero* had increased hsCRP and a trend towards a pro-atherogenic lipid profile. These observations may be indicative of an increased risk of atherosclerosis in later life and longitudinal studies are warranted. We hypothesise that our early assessment of aIMT may have precluded time for structural change to the artery following exposure late in pregnancy, which should be taken into account during future investigations. Subsequent inflammatory insults during childhood and early adolescence in individuals exposed to chorioamnionitis *in utero* may affect the progression of atherosclerosis. Understanding the initiation and progression of atherosclerosis in early life will allow the development of strategies to

alter cardiovascular risk trajectories and ultimately prevent morbidity and mortality associated with atherosclerosis.

Conflict of Interest

The authors would like to declare no conflicts of interest.

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Abbreviations

Analysis of variance (ANOVA)

Aortic intima-media thickness (aIMT)

Blood pressure (BP)

Carotid IMT (cIMT)

General Electric (GE)

Gestational age (GA)

Hematoxylin and eosin (H&E)

High-density lipoprotein (HDL)

Low-density lipoprotein (LDL)

Preterm premature rupture of membranes (PPROM)

Royal Women's Hospital (RWH)

Serum high sensitivity C-reactive protein (hsCRP)

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Tables

Table 1. Population description and pregnancy outcomes

	Cases (n=20)	**Controls (n=31)**	***P*-value[b]**
Maternal age (years)[a]	32.25 (6.42)	32.06 (4.41)	0.90[c]
Nulliparous	6 (30%)	15 (50%)	0.24
PPROM	15 (75%)	14 (45%)	0.08
Antibiotics received during pregnancy	17 (85%)	11 (35%)	<0.01
Antibiotics received during labour	13 (65%)	11 (35%)	0.08
Obstetric diagnosis of chorioamnionitis	10 (50%)	2 (6%)	<0.01
Organisms present on vaginal swab	15 (75%)	9 (29%)	<0.01
Mode of Delivery			
Vaginal (vertex)	10 (50%)	11 (35%)	0.39
Vaginal (breech)	1 (5%)	1 (3%)	1.00
Caesarean (in labour)	1 (5%)	0 (0%)	0.40
Caesarean (not in labour)	6 (30%)	17 (55%)	0.09
Forceps	2 (10%)	1 (3%)	0.56
Ventouse	5 (5%)	1 (3%)	0.03
Highest level of resuscitation			
Cardiac compressions	1 (5%)	0 (0%)	0.40
CPAP / PEEP	4 (20%)	11 (35%)	0.35
Face mask ventilation	6 (30%)	9 (29%)	1.00
None	9 (45%)	11 (35%)	0.57
Gestational age (completed weeks)[a]	32.35 (1.81)	32.61 (1.80)	0.61[c]
Male births	16 (80%)	12 (39%)	0.01

Birth weight (g)[a]	2027.20 (451.63)	1999.74 (385.84)	0.82[c]
Birth weight z-score[a]	0.46 (0.98)	0.37 (0.80)	0.71[c]
Apar score at 1 minute[a]	6.60 (2.28)	7.29 (1.90)	0.25[c]
Apar score at 5 minutes[a]	8.20 (1.54)	8.35 (0.88)	0.65[c]
Newborn head circumference[a]	30.44 (2.08)	30.52 (1.52)	0.88[d]
Newborn pondral index[a]	0.02 (0.003)	0.02 (.005)	0.13[d]

[a]Mean (SD)

[b]*P*-values from Fisher's exact tests, unless otherwise specified

[c]*P*-values from Student's t-tests

[d]*P*-values from Welch's test

Table 2. Serum biochemistry measurements based on presence / absence of chorioamnionitis

Serum biochemistry	**Cases (n=19)**	**Controls (n=29)**	**Mean difference (95% CI)**	***P*-value[b]**
High sensitivity CRP (mg/L)	2.93 (4.23)	0.45 (0.45)	-2.48 (-4.52, -0.44)	0.02
Total cholesterol (mmol/L)	3.31 (0.85)	3.18 (0.85)	-0.12 (-0.63, 0.38)	0.62
HDL cholesterol (mmol/L)	0.87 (0.38)	1.03 (0.29)	0.16 (-0.03, 0.35)	0.10
LDL cholesterol (mmol/L)	1.82 (0.76)	1.69 (0.78)	-0.13 (-0.59, 0.33)	0.57
Triglyceride (mmol/L)	1.19 (0.76)	0.95 (0.34)	-0.24 (-0.62, 0.14)	0.21

Data presented are mean (SD)

[b]*P*-values from Student's t-tests

Table 3. Serum biochemistry, abdominal ultrasound and blood pressure measurements based on severity of choriomanionitis

	Severity of chorioamnionitis			
Serum biochemistry	**Maternal (n=5)**	**Fetal (n=14)**	**Control (n=29)**	***P*-value[a]**
Total cholesterol (mmol/L)	3.00 (0.19)	3.42 (0.97)	3.18 (0.85)	0.57
High sensitivity CRP (mg/L)	3.86 (7.72)*	2.60 (2.45)	0.45 (0.45)*	<0.01
HDL cholesterol (mmol/L)	0.84 (0.52)	0.88 (0.32)	1.03 (0.29)	0.26
LDL cholesterol (mmol/L)	1.47 (0.23)	1.95 (0.85)	1.69 (0.78)	0.42
Triglyceride (mmol/L)	1.45 (1.04)	1.09 (0.65)	0.95 (0.34)	0.16
Abdominal ultrasound	**Maternal (n=6)**	**Fetal (n=14)**	**Control (n=31)**	***P*-value[a]**

Mean minimum aIMT (mm)	0.34 (0.07)	0.36 (0.08)	0.36 (0.07)	0.87
Mean maximum aIMT (mm)	0.48 (0.09)	0.48 (0.09)	0.50 (0.08)	0.80
Mean average aIMT (mm)	0.40 (0.07)	0.42 (0.08)	0.42 (0.07)	0.78
Mean systolic BP (mm Hg)	75.00 (15.59)	68.14 (12.78)	68.74 (11.20)	0.53
Mean diastolic BP (mm Hg)	50.00 (10.95)	45.86 (16.76)	42.29 (10.26)	0.37
Mean average BP (mm Hg)	58.40 (11.26)	50.79 (10.72)	51.52 (10.35)	0.36

Data presented are mean (SD)

[a]*P*-values from ANOVA comparing three groups

*Significant difference between Maternal and Control groups (*P*=0.04) using Scheffe test

Abbreviations: aIMT, aorta intima media thickness; BP, blood pressure; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Table 4. Abdominal ultrasound and blood pressure measurements based on presence / absence of chorioamnionitis

	Cases (n=20)	**Controls (n=31)**	**Mean difference (95% CI)**	***P*-value[a]**	**Adjusted mean difference (95% CI)**	***P*-value[b]**
Mean minimum aIMT	0.35 (0.08)	0.36 (0.07)	-0.01 (-0.05, 0.04)	0.79	-0.01 (-0.05, 0.06)	0.82
Mean maximum aIMT	0.48 (0.09)	0.50 (0.08)	-0.02 (-0.06, 0.03)	0.50	-0.01 (-0.07, 0.04)	0.63
Mean average aIMT	0.41 (0.08)	0.42 (0.07)	-0.01 (-0.06, 0.03)	0.60	-0.00 (-0.05, 0.05)	0.88
Mean systolic BP (mm Hg)	69.95 (13.47)	68.74 (11.20)	1.21 (-5.88, 8.29)	0.73	2.89 (-4.89, 10.68)	0.46
Mean diastolic BP (mm Hg)	46.95 (15.27)	42.29 (10.26)	4.66 (-2.60, 11.91)	0.20	6.20 (-1.92, 14.33)	0.13
Mean average BP	52.79 (11.09)	51.52 (10.35)	1.27 (-4.96, 7.50)	0.68	2.99 (-3.96, 9.94)	0.39

Data presented are mean (SD); CI = confidence interval

Abbreviations: aIMT, aorta intima media thickness; BP, blood pressure

[a]*P*-values from an unadjusted single model linear regression

[b]*P*-values from a single model multivariate regression adjusted for birth weight z-score, PPROM, infant sex and minimum aortic lumen diameter