ENAMEL NEONATAL LINE THICKNESS IN DECIDUOUS TEETH OF AUSTRALIAN CHILDREN FROM KNOWN MATERNAL HEALTH AND PREGNANCY CONDITIONS

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Declarations of interest
None

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ABSTRACT

Background

Physiological disruptions to early human development have implications for health and disease in later life. Limited research has explored how prenatal factors influence dental development in children of mothers with known pregnancy conditions. Enamel in human deciduous teeth begins forming *in utero* and is highly susceptible to physiological upsets experienced perinatally. The moment of birth itself is marked in deciduous enamel by the Neonatal Line (NNL) as a baby transitions from the uterine to external environment. This study evaluates the effect of maternal health factors that include stress and alcohol consumption on NNL in teeth from Australian children.

Study design and subjects

Mothers (n = 53) were interviewed about their health during pregnancy and experience of birth. Sixty-five deciduous teeth (incisors, molars, one canine) from their children were donated for histological examination. Neonatal line thickness was measured from thin sections and evaluated against maternal and neonatal factors using statistical analyses, controlling for tooth type and birth number.

Results and conclusions

The only maternal factor of a statistically significant effect on NNL thickness was alcohol consumption. Children of mothers who drank occasionally during pregnancy had a thicker NNL when compared to children of mothers who abstained. These results suggest that maternal lifestyle factors influence NNL formation possibly due to physiological changes that disrupt calcium homeostasis during enamel deposition. We highlight large intra-specific variation in human NNL expression. The potential of dental sampling in identifying children with prenatal exposure to alcohol is suggested.

Keywords: enamel, dental histology, neonatal line, alcohol, prenatal, perinatal, postnatal

Highlights:

- A microscopic deciduous tooth “birth marker” is known as the Neonatal Line (NNL)
- NNL can be used to study a baby’s pre- and perinatal environment
- Results show prenatal exposure to alcohol has the strongest effect on NNL
- Other maternal health factors are not linked to NNL in our sample
- We highlight a large extent of intra-specific variation in NNL expression

1. Introduction

The effect of maternal health and environment during pregnancy on foetal development has been well documented [e.g. 1-4], and can be explained using the Developmental Origin of Health and Disease (DOHaD) approach [5]. The DOHaD framework indicates foetal origins of adult health and disease, and has received support from observations in epidemiology, psychobiology, and epigenetics amongst other disciplines [6]. For example, it has been shown that exposure to nutritional deficiencies and/or medicinal drugs can impact early foetal development and programming, impacting morbidity later in life [7]. Poor maternal condition, which may be due to exposure to stress [8, 9], very young or very advanced maternal age [10 – 12], smoking in pregnancy (see [13, 14]) and illness (see [15]), have also been linked to negative pregnancy outcomes including low birth weight, pre-term birth and even infant mortality indicating a strong relationship between maternal health and condition and foetal outcomes. Prenatal alcohol exposure has been linked with congenital anomalies of the developing foetus affecting the brain [16, 17]. As the DOHaD approach has implications for strategies in identifying adults most at risk of developing certain conditions, the research efforts are ongoing and global [18]. For example, new DOHaD working groups have been recently established in Australia in New Zealand [19]. Evidence exists that the skeletal system of a developing baby can be affected by maternal health, lifestyle, and pregnancy conditions, determining adult life bone mineral density (BMD) [20], and overall bone health [21 - 23]. Less understood is the effect of these maternal factors on dental enamel characteristics *in utero* in different populations.

1.1. *Dental enamel microstructure*

Children's teeth preserve very well when stored in hospitals, by dentists, or by parents at homes, because of their high mineralisation content [24, 25]. They form a valuable source of early human developmental data, especially when combined with documented health records of a

baby and its mother [26 - 29], allowing to test hypotheses within the DOHaD framework. Dental enamel forms through amelogenesis, which is a tightly controlled and rhythmical process of enamel deposition during dental development [30, 31]. As enamel is laid down in regular increments, it leaves behind a microscopic record of tooth growth [25]. When physiological or external factors disturb this process, it is manifested as a localised reduction in enamel density that can be detected microscopically [32, 33]. Deciduous teeth thus serve as a long-lasting record of developmental homeostasis and its abnormalities, as they do not change once fully formed and then lost [34].

At the histological level, dental enamel is constructed from incremental lines which are evidence of a biorhythm [30, 31]. These increments are divided into daily (deposited every 24 hours) and longer-period (6 - 12 days) lines [35]. The longer-period markers are known as Retzius lines, which form when enamel secretion slows down temporarily, and at regular intervals [36]. One type of a Retzius line is the neonatal line (NNL) which forms at the time of birth [37, 38]. It indicates an intersection between pre- and post-natal enamel in a newborn's deciduous (and the first permanent molar) teeth [25, 34]. The NNL forms as a result of a disruption to an otherwise regular amelogenesis as a baby leaves the intrauterine environment. Disturbances to human enamel development can manifest both macroscopically (e.g. linear enamel hypoplasia) and microscopically (accentuated markings) (see [25]). However, the aetiology of these is usually difficult to diagnose specifically. Studies have matched them to several different factors that include malnutrition, disease, and consumption of toxins [e.g. 39 - 42]. Therefore, the NNL can be considered a physiological indicator of a disrupted environment as a new born baby passes from *in utero* to postnatal phases of growth.

1.2. *Neonatal line and birth*

Studying the NNL has seen applications in several areas of research, including early human development, biological anthropology, and forensics [e.g. 43 - 52], highlighting its great

potential as a suitable "birth marker" retained in teeth. Studies have used the NNL to determine pre- and post-natal life stages of infant survival [43, 44]; reconstruct prenatal and childhood mortality, morbidity, and development in past human populations [44, 45]; map the timing of enamel development [46 - 48]; and make conclusions about still, premature, and full term births [49, 50]. The relationship between NNL and birth timing, birth weight, delivery mode and gestation length has also been reported [26 - 29, 51 - 52]. However, conflicting results indicating links between birth condition and the NNL characteristics are apparent. For instance, NNL thickness appeared to reflect delivery mode (normal birth, still birth, Caesarean section) in Turkish children [26], but it did not in a sample of Italian children [28]. This discrepancy may signal intra-specific and inter-populational variation in how the NNL is expressed in modern teeth representing different modes of pregnancy and baby delivery. The conclusion has been that the NNL appears thicker in babies born by complicated delivery, and thinner in those from Caesarean sections [26, 50]. A prolonged "slowing down" of enamel deposition during the birth phase could plausibly explain this observation. More data are needed to further explain this, considering prior suggestions that NNL thickness may be more related to gestation related physiological changes, alongside hypocalcaemia (reduction in blood serum calcium), than delivery mode [28]. The mineralisation of deciduous enamel has indeed been found to vary substantially in children from pre-term pregnancies [52].

The aim of this study is to investigate the extent to which a series of *in utero* maternal conditions may affect the thickness of the NNL. We evaluate the NNL in relation to maternal sources of stress, medical history, maternal age and offspring health and lifestyle, as well as birth conditions such as mode of delivery and length of labour. We address this question for the first time in an Australian sample, shedding new light on its maternal health and dental response to *in utero* environments.

2. Materials & Methods

This study involved two phases. Firstly, dental samples from living children were collected and their mothers were interviewed about birth and health during pregnancy. Secondly, the tooth donations were processed for histological examination. This research was approved by the Humanities and Social Sciences Delegated Ethics Review Committee (DERC) at the Australian National University, Canberra, Australia (Protocol 2018/019).

2.1. *Interviews of mothers*

Commencing in March 2018, a call for tooth donations and interviews was advertised through a local society ("Canberra Mums") for mothers based in Canberra, Australia. A total of 53 mothers responded, all of whom were residents in Canberra or the neighbouring city of Queanbeyan. The participating mothers were asked to provide a milk tooth from each child they wished to include in our study. For each child, they completed an interview (either in person or over the phone) answering a series of questions regarding their health and circumstance during pregnancy and birth, along with information about age and ancestry (a list of key questions is shown in **Table 1**). Most of the data were obtained from medical records kept by the mothers, though some were based off recall. While this could potentially add error to our results, previous studies have found high levels of accuracy in recall of factors associated with pregnancy and birth [53, 54]. The identity of all mothers was kept anonymous and each child was assigned an ID number. Each mother gave consent to use the data prior to the interview. By the end of the project, all participating mothers were given a false coloured image of their child's tooth cross section as a token for contribution to the project.

2.2. *Dental histology*

Teeth were analysed as blinded samples and processed into thin sections using standard methods appropriate for human deciduous dentition [47-49]. All procedures took place in the

School of Archaeology and Anthropology Histology laboratory at the Australian National University in Canberra, which is also where the thin sections are curated. There were 65 tooth donations in total, with n = 59 incisors, n = 5 molars, and n = 1 canine. The specific tooth types included: Ldi_1 n = 10, Ldi^1 n = 12, Ldi_2 n = 7, Ldi^2 n = 3, Ldm^1 n = 1, Rdc^1 n = 1, Rdi_1 n = 8, Rdi^1 n = 12, Rdi_2 n = 2, Rdi^2 n = 5, Rdm_1 n = 1, Rdm^1 n = 1, Rdm_2 n = 1, Rdm^2 n = 1. All teeth were non-carious, non-restored, and of no to minimal macro-wear. Each tooth was sterilised in 75% ethanol [55] before subsequent embedding in epoxy resin. Using a low speed Kemet Micracut® 151 Precision Cutter with a Diamond cutting Disc of 150 mm diameter, the embedded blocks were sectioned longitudinally through the teeth to reveal histology surfaces. The incisors and canine were cut in a labial-lingual plane through the middle of the cusp tip all the way to the tooth cervix [48]. The molar sections were taken in a bucco-lingual plane cutting through mesial cusp tips and dentin horns [47, 56]. Section obliquity (see [57]) was minimised as much as possible by marking the sectioning location on tooth surfaces prior to embedding to match exact positioning on the saw. Dentin shape was also evaluated [58]. Sections were then mounted onto glass slides, gradually ground on 200 mm grinding pads (Buehler® SiC Abrasive Paper, plain) of P400 and P1200 grit size to reach approximately 100 μm (± 15 μm) section thickness to reveal the NNL. Ground sections were then polished using Buehler® MicroPolish II 0.3. μm powder on a wet Buehler® Polishing cloth. Samples were cleaned in an ultrasonic bath, dehydrated in ethanol and cleared in xylene before cover-slipping.

Imaging of the thin sections was undertaken using an Olympus BX53 high powered microscope equipped with an Olympus DP74 camera. Olympus CellSens® 2018 software was used to capture images at 40x magnification. The NNL thickness (i.e. width measured from 2D sections) data were collected from images analysed in ImageJ® software. Similarly to Canturk and colleagues' methods [26], the average thickness of NNL was calculated from three points along a NNL in each tooth. The points were located by dividing the total NNL length by three

to estimate equal NL segments. A midpoint of each segment was then measured. This ensured consistent measurements regardless of the NNL position within the enamel crown (i.e. closer to the tip, mid-crown, closer to the cemento-enamel junction). As the measurements were taken by one observer (JJM), an intra-observer error evaluation was conducted on seven, randomly selected images to ensure repeatability of the data (checked using a Wilcoxon *W* signed-rank test).

2.3. *Statistical analysis*

All data were analysed in IBM SPSS Statistics 25. Sample size dependent, data normality was assessed using either a Shapiro Wilk or Kolmogorov-Smirnov test. The hypothesis that the NNL thickness should be influenced by maternal health, pregnancy and birth conditions was evaluated using three types of tests set at $p = 0.05$ (Bonferroni corrected where necessary) (see **Table 1** for independent variables). Firstly, Spearman's *Rho* or Pearson's *r* correlations were used to assess correspondence between NNL thickness and mother's height, maternal age at pregnancy, maternal age at birth, gestation, labour length, birth weight, and birth height. Secondly, Mann-Whitney *U* (two comparisons) or Kruskal-Wallis *H* (> two comparisons), or independent samples *t*- tests were conducted to evaluate potential differences in NNL thickness between the sexes of infants, number of births, past miscarriages, pregnancy alcohol consumption, pregnancy illness, pregnancy stress, delivery method, and induction. Finally, a Wilcoxon *W* signed-rank test was performed to compare teeth from cases of siblings from the same mother.

There were several different tooth types donated for the study which necessitated performing the analyses over several steps. The five molars and one canine had to be excluded from repeated analyses as meaningful inferential analysis could not be performed on such a small sample size. Some mothers also donated teeth for more than one child (up to three children), and delivered their babies outside Australia. Therefore, the analyses were conducted on the

entire sample first. Second, in order to avoid confounding the overall analysis by including siblings (i.e. related samples), each set of tests was further repeated including only one child from mothers with multiple children, and on children only born in Australia.

3. Results

Descriptive data for mothers and their children are presented in **Tables 2** (quantitative) and **3** (qualitative). Results from the inferential analysis can be found in **Tables 4** and **Supplement Table 1**. There were no statistically significant differences between the intra-observer repeated measurements of NNL thickness (n = 7, $W = 9.000$, $p = 0.753$).

3.1. *Descriptive results*

Mean age at pregnancy for the whole sample was 29.4 years old (SD = 5.04) with a mean maternal age at birth of 29.9 (SD = 5.52). The age range of children was 6 to 30 years of age. We note that we interviewed 53 mothers, but we present the data per child (i.e. out of 65, although not all questions were answered for all children – see **Table 3** for clarification). The country of pregnancy experienced by mothers of 62 children (95%) was Australia, with the rest of the mothers self-reporting Asian and European countries. However, Australia as the country of birth was reported to represent 56 children (86%), with the remaining mothers delivering overseas. Therefore, our sample predominantly represents pregnancies and births experienced in Australian contexts. Canberra was the city of residency for mothers of 52 children (80%), with other Australian and overseas cities, and some urban and rural locations reported for the remainder of the sample. Therefore, most of the sample can be considered to represent urban Australia. Fifty teeth represent first born children (77%). One past miscarriage was experienced by mothers of 17 (30%) children. Occasional alcohol consumption during pregnancy, which ranged from one standard drink per week to one standard drink per month, was self-reported by mothers of ten children (16%). Mothers of twenty four (37%) and 29 (45%) children reported

experiences of stress and illness during pregnancy respectively. Of the stress experienced by mothers, 33% said it was ongoing through the entire pregnancy. Of the remaining mums, 42% said they felt it through the first trimester (42%), followed by 12.5% of mums feeling stress only in the third trimester and 8.3% only in the second trimester.

The donated teeth were from 38 (58%) girls and 27 (42%) boys. There were 36 (55%) teeth representing one child per mother, but 29 (45%) teeth came from siblings. Twenty eight of the latter included 14 teeth representing the first born and 14 teeth the second born child. Only one mother donated teeth for three children, which means our sample also includes one tooth from a third born. The NNL was not clearly visible in seven sections (**Table 3**). Where the NNL was clearly identified (**Table 4**), its average thickness was 10.28 μm (SD 6.36) across the whole sample. Once sub-divided by tooth type, mean NNL thickness was 10.92 μm (n = 52, SD 4.90 μm) in incisors, 16.40 μm in molars (n = 5, SD 9.38), and 18.43 μm in the one canine.

3.2. *Inferential results*

The inferential analysis yielded an almost complete lack of statistical significance on the effect of different maternal and neonate characteristics on the NNL thickness, except for a consistent relationship with alcohol consumption during pregnancy.

Firstly, the results of correlations between NNL thickness and mother's height, maternal age at pregnancy, maternal age at birth, gestation, labour length, birth weight, and birth height returned weak *r* and *Rho* coefficients and $p > 0.05$ almost entirely across the board (**Supplement Table 1**). Secondly, non-parametric comparisons of NNL thickness between different groups of infant sex, number of births, past miscarriages, pregnancy alcohol consumption, pregnancy illness, pregnancy stress, delivery method and induction also returned no statistically significant results, except for the effect of alcohol consumption during pregnancy (**Table 4**).

When narrowing the sample down to incisors within mothers with pregnancy and birth experiences in Australia only, and excluding related samples, all first born children (second and third sibling excluded, n = 39, $U = 200.000$, $p = 0.001$; $n_{alcohol} = 7$, mean = 16.37µm, SD = 4.62, $n_{none} = 32$, mean = 7.92 µm, SD = 5.56), and then with the third child included (n = 28, $U = 98.000$, $p = 0.013$; $n_{alcohol} = 5$, mean = 16.29µm, SD = 5.64, $n_{none} = 23$, mean = 8.67 µm, SD = 5.61) had thicker neonatal lines from mothers who reported occasional alcohol consumption (**Figures 1, 2**). This result was consistent when further excluding teeth that did not show a NNL when considering second and third sibling excluded (n = 32, U = 151.000, $p = 0.002$; $n_{alcohol} = 7$, mean = 16.37µm, SD = 4.62, $n_{none} = 25$, mean = 10.13µm, SD = 4.07), and the sub-group that included the third sibling (n = 24, U = 78.000, $p = 0.030$; $n_{alcohol} = 5$, mean = 16.29µm, SD = 5.64, $n_{none} = 19$, mean = 10.50µm, SD = 5.64). In all these cases we report the adjusted exact significance. Considering the simultaneous multiple testing in this category (eight independent variables including alcohol consumption), the Bonferroni corrected $p = 0.006$ sustains the statistical significance of our finding in the 39 and 32 incisors as above.

Finally, a Wilcoxon *W* signed-rank test comparing teeth from cases of siblings from the same mother of different pregnancy experiences resulted in no statistically significant results either (n = 12, $W = 15.000$, $p = 0.060$).

4. Discussion

The aim in this study was to investigate the effect of maternal health and pregnancy condition on the formation of a "birth marker", known as the NNL, in children's deciduous teeth. The NNL has long been known to reflect a temporary slowing down of enamel formation at the time of birth, marking the transition from a pre- to post-natal environment [34, 37]. Previous research has indicated that the NNL expression may be associated with delivery mode and gestation [29, 50, 51], but, to the best of our knowledge, no attempts to investigate maternal lifestyle factors potentially relating to NNL during pregnancy have been undertaken in an Australian sample.

Overall, our study did not find clear links between NNL thickness and pregnancy conditions (gestation, labour length, number of births, delivery method, induction), neonatal traits (infant sex, birth weight, birth height), and maternal factors (past miscarriages, mother's height, maternal age at pregnancy, maternal age at birth, pregnancy illness, pregnancy stress). However, we identified a statistically significant effect of occasional consumption of alcohol on NNL thickness. The children of mothers who drank occasionally during pregnancy had a thicker NL when compared to the children of mothers who abstained. Our findings indicate two key points for discussion. Firstly, in agreement with previous studies and reviews examining alcohol consumption during pregnancy and foetal and life course outcomes [59-62], alcohol had a negative effect on a child's dental enamel formation at birth in our study. Secondly, there appears to be a large extent of variation within the maternal experience of pregnancy and how it links to deciduous NNL formation.

4.1. *Prenatal exposure to alcohol*

Despite the lack of obvious relationships between maternal health and pregnancy variables and NNL thickness, the present study did find a strong association with one lifestyle factor - the occasional consumption of alcohol while pregnant. The NNL in children from drinking mothers was significantly thicker compared to those found in children whose mothers abstained. As enamel forms, it is highly sensitive to physiological disturbances, including nutritional issues, disease, even psychological trauma [25, 32, 63 - 65], that can be captured within its increments. Likely dependent on the severity of stress, the enamel secretions cells (ameloblasts) can respond to stress at different thresholds [33]. The most extreme can be a complete temporary cessation of enamel growth. The variation in NNL thickness of the NNL could thus be linked to type and severity of stressors as enamel enters the post-natal environment.

In light of the literature reporting on the effects of smoking and drinking alcohol on a developing foetus [13, 14, 66 - 72], it is perhaps no surprise that developing teeth would be

affected by occasional alcohol consumption as well. For instance, moderate drinking (defined as < 40 g alcohol/ day) during pregnancy has been previously linked to congenital disorders, intrauterine growth restriction, and behavioural difficulties in children [66]. When alcohol consumption approximated 100g a week, newborns' head circumference and weight were also reduced [66]. However, methodological limitations surrounding the definition and measures of "light", "moderate", and "heavy" drinking have long complicated conclusions about the associations between alcohol and the foetus [67]. Indeed, in a study analysing alcohol ingestion before and throughout pregnancy in a cohort of Italian mothers, consuming more than three drinks while pregnant was associated with pre-term delivery [68]. This was, however, not the case when considering one or two drinks [68]. Australia based research investigating long term effect of prenatal exposure to alcohol identified the development of mild chronic kidney disease in children aged 30 years old [69]. Taken together, the DOHaD understanding of developmental conditioning on health and disease clearly plays a role in determining health and disease. The mechanisms explaining alcohol-induced responses on the foetus need further elucidation. Though pre-term births linked to prenatal alcohol exposure may be due to prostaglandins mediating parturition [70].

Extending to skeletal tissue development, experimental research into calcium metabolism using animal models shows that maternal consumption of ethanol leads to a reduction in mineral in bone [71], decreasing foetal growth and maturation of the skeleton *in utero* [72]. In addition, dental loss and development of periodontal disease have been noted to occur in individuals who habitually consume alcohol, indicating its influence on the human skeletal system both in teeth and bone (weakening jaw bone stability) [73, 74]. Most recent research suggests that alcohol may act as a suppressant of genes regulating calcification in the stem cells of dental pulp [75]. Developmental biology experiments on adult female pregnant mice subjected to alcohol indicate offspring of reduced weight, decreased mandible size, restricted skull and mandible

growth, and delayed tooth germ formation, calcification, and dental eruption [76]. These studies support the response of dental tissues to some form of foetal stress, and hence agree with the thicker NNL data in the children exposed to alcohol prenatally in our study.

The lack of clear relationships between variables that related to other neonatal features in our study were also noted previously when specifically the NNL [28] or other dental defects were considered [79]. For instance, despite pre-term labour and exposure to antibiotics did not relate to dental stress, low birth infants had a significantly increased record of enamel hypoplastic defects when compared to those of normal birth, in a Brazilian sample of children [79]. Taken together, the findings in our study can be interpreted to indicate that the NNL may not reach its full recovery potential as quickly as it does in children from abstaining mothers. It appears that maternal lifestyle may have an influence on the NNL, fitting within the DOHaD framework. Epidemiological and epigenetic data investigating the effect of social behaviour on foetal development and impacting the life course from the perspective of hard and soft tissue regulation indeed support this suggestion [22, 23].

To the best of our knowledge, ours is the first study to investigate NNL thickness and alcohol in humans. Future research will need to secure more samples and extend to other populations to validate the ways in which dental histology responds to prenatal alcohol exposure. Incorporating the assessment of enamel mineralisation [52], 3D measurements, elemental mapping of trace elements at the NNL [80], into 2D thin sectioning may also prove more fruitful. Interpretations of our finding are further limited by the lack of information on the exact dose of alcohol consumed by the mothers, and the timing of alcohol exposure by the developing baby. Further research may build upon our results incorporating these variables into study design. The outcomes of such research may lead to improving current efforts in identifying children who may have been exposed to alcohol prenatally. There is potential for sampling

children's lost teeth as part of intervention efforts when dealing with those who experience internalising and externalising behaviours related to parental alcoholism [81, 82].

4.2. *Intra-specific variation*

This study measured multiple different variables that related to maternal pregnancy health and condition, the health of neonates. Intriguingly, no strong association between these and the NNL in children's teeth was observed. This result adds further data to some of the currently conflicting conclusions from similar studies. For example, Canturk and colleagues [26] reported the NNL to be thicker in dental samples from normal birth conditions, but thinner in the Caesarean samples, while still births showed no evidence of the NL. On the other hand, a study by Zanolli and colleagues [28], which examined teeth in 100 living children, found no difference in NNL thickness for Caesarean vs. natural births. Zanolli and colleagues [28], however, highlighted that there are multiple other factors that relate to gestation and birth dynamics underlying the NNL expression. Indeed, the analyses in the present study demonstrate that these relationships are more complex than previously thought, with the multiple maternal variables measured here mostly not relating to NNL at all (at least statistically).

Outside of our study limitations that include sample size (i.e. particularly when comparing alcohol consumption groups), the present study likely highlights that the human maternal and foetal nexus may not always manifest in the developing teeth of children. Indeed, as also shown in previous NNL research [28, 37], histology in some of the teeth studied here did not show clearly visible lines, whereas other teeth appeared to display lines of increased thickness. As noted by Canturk et al [26], still births in their study were associated with a lack of NNL. This can be plausibly explained by the absence of environmental disruption to enamel deposition at birth. As all of the children in our study were born alive, it becomes clear that there is a broad spectrum of NNL expression in children from mothers of varied experiences of pregnancy and birth conditions. This could relate to individual level susceptibility (e.g. immunity, genetic

predisposition) of dental enamel to record disturbances [25, 83]. The thickness of the NNL itself may relate to enamel secretion rates that have been shown to accelerate and slow down in different parts of the tooth [84]. Recent research indicates an association between its micro-morphology and tooth type [85].

Finally, our findings raise a point that is worth considering within practice where the NNL identification is used in forensic scenarios [e.g. 86-89], and reconstruction of life histories in ancient contexts [e.g. 90-92]. When maternal health and other contextual background is not available, the dental samples on their own potentially offer a limited insight into one's biology when alive. Carefully controlled studies that access medical and health records [e.g. 64, 84] are able to better match the external factors with tooth microscopic features. Studies such as ours, and in combination with conflicting results from previous research in modern children [26, 28], show the vast extent of variation in extant humans which may help in future interpretations of non-experimental data.

5. Conclusion

This study is the first to investigate the microscopic thickness of the NNL in teeth from Australian children, examined in relation to pregnancy conditions (gestation, labour length, number of births, delivery method, induction), neonatal traits (infant sex, birth weight, birth height), and maternal factors (past miscarriages, mother's height, maternal age at pregnancy, maternal age at birth, pregnancy illness, pregnancy stress, alcohol consumption). The results shed new light on the nature of the relationship between external factors and dental enamel formation, contributing to ongoing research into physiological stress in human biology.

The study reports that occasional consumption of alcohol by pregnant women prolongs a temporary disruption of their children's enamel formation. However, it also demonstrates that the reconstruction of human *in utero* conditions from NNL is more complex than previously

thought. We highlight variation of the pregnancy experience and its influence on hard tissue growth in the living. Seeing as dental histology is often used in forensic and ancient human research, we contribute new data illustrating the broad spectrum of NNL manifestation. This may assist in future interpretations in these disciplines. It is recommended that research investigating the maternal-foetal links in relation to dental micro-anatomy pays particular attention to maternal lifestyle factors. Addressing questions within the DOHaD framework that focus on prenatal alcohol exposure may benefit from incorporating dental sampling into study design.

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(*hidden for the purposes of blinded review*)

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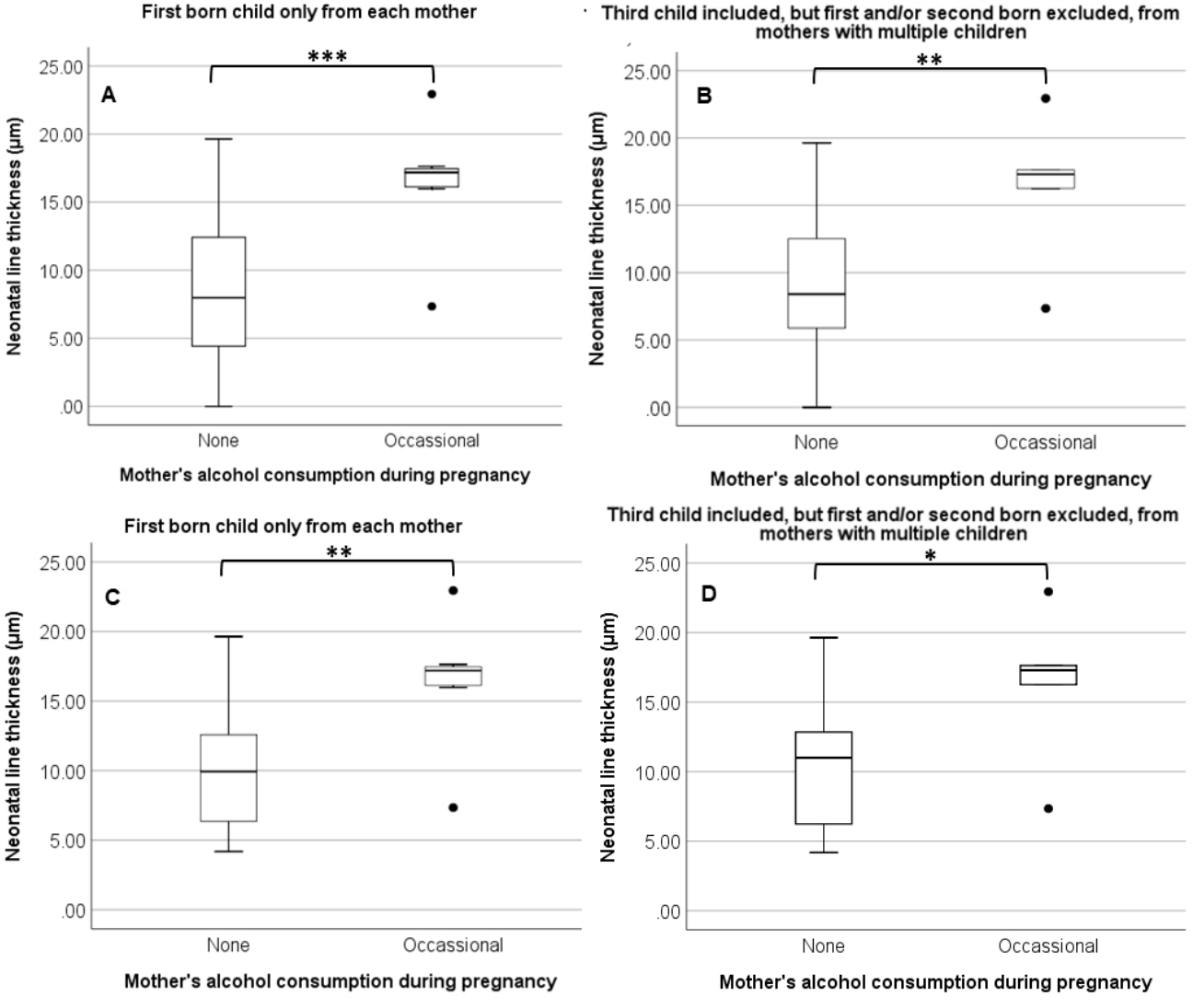
Figure 1. Box plots showing stark differences in neonatal line thickness in the incisors of Australian children from mothers who self-reported occasional consumption of alcohol and abstinence. The plots A and B report data from all teeth, with A including the first born only, and B including the third born from one mother with multiple children. The plots C and D report data from all teeth excluding those without a NNL.

Figure 2. Summary of the main finding in the present study whereby children's teeth from mothers who consume alcohol occasionally appear to deposit a thicker neonatal line in their deciduous incisors, indicating prolonged disruption to enamel formation at the time of birth. In order to protect participant identity, this illustration is schematic - the image of enamel shown

in the middle of the figure was selected at random and is only shown to indicate NNL micro-morphology.

CHILD'S INCISOR NEONATAL LINE THICKNESS AND MOTHER'S ALOCOHOL CONSUMPTION DURING PREGNANCY



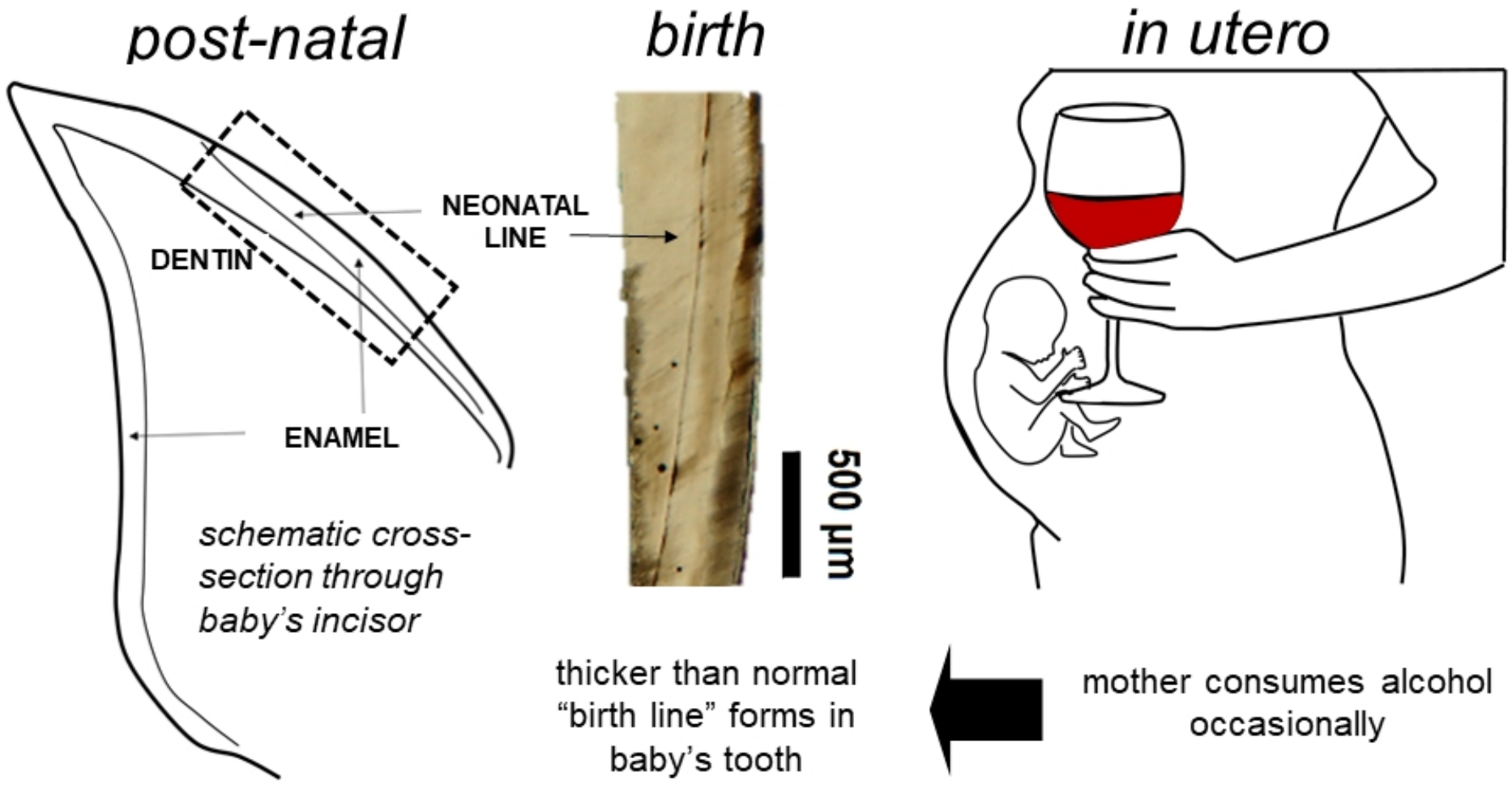

post-natal
birth
in utero
NEONATAL LINE
DENTIN
ENAMEL
schematic cross-section through baby's incisor
500 µm
thicker than normal "birth line" forms in baby's tooth
mother consumes alcohol occasionally

Table 1. Key questions relating to maternal health, pregnancy and birth experience asked in the interviews with participating mothers in this study.

Questions	Question type	Focus variable
What is your height?	Open ended	Mother's height*
At what age did you fall pregnant (in years)?		Maternal age at pregnancy*
Was this your first pregnancy? If no, what number pregnancy was it?		Number pregnancy**
At what age did you give birth (years)?		Maternal age at birth*
What was the gestational age of your child at birth (weeks)?		Gestation*
What was the weight of your child at birth (kg)?		Birth weight*
What was the length of your child at birth (cm)?		Birth height*
Did all previous pregnancies result in live births?		Past miscarriage**
Did you consume any alcohol during pregnancy (Y/N)? If yes, in which trimester and how often?	Dichotomous and open ended	Alcohol**
Did you suffer any of the following health conditions during your pregnancy (Y/N)? If yes, at approximately what month gestation and for what duration?: gestational diabetes, high blood pressure, severe morning sickness, hyperemesis, gravidarum, other.		Illness**
Did you experience any significant stress during your pregnancy (Y/N)? If yes, at what gestational month did this occur? If yes, what was the stress caused by?		Stress**
Did you use any pain management (Y/N)? If yes, what forms of pain management did you use (i.e. gas, epidural etc.). Did you (circle one): deliver naturally without any assistance, deliver with assistance of vacuum of forceps, progress through labour to an emergency caesarean section, have a planned caesarean section.	Dichotomous and multiple choice	Delivery method**
Was your labour induced (Y/N)? If yes, what method was used?	Dichotomous and open ended	Induction**
What was the sex of your child?	Open ended	Sex of infant**
How long was your labour (hours)?		Labour*

*Quantitative data **Qualitative data (for analysis purposes)

Table 2. Quantitative descriptive data for mothers and infants.

Maternal and infant variables	N	Min.	Max.	Mean	SD
Maternal age at pregnancy (years)	65	17.00	40.00	29.38	5.04
Maternal age at birth (years)	65	12.00	41.00	29.85	5.52
Gestation (weeks)	65	28.00	42.00	39.17	2.23
Labour length (hrs)	56	2.00	72.00	15.94	13.49
Birth weight (g)	65	1320.00	4800.00	3268.23	577.95
Birth height (cm)	57	34.50	58.00	50.15	3.38
Mother's height (cm)	64	150.00	178.00	165.83	6.14
Neonatal line (NNL) thickness (µm)					
All teeth	65	0.00	31.52	10.28	6.36
Molars	5	8.35	31.52	16.40	9.38
Canine	1	-	18.42	18.42	18.42
Incisors	59	0.00	22.94	9.63	5.82
Incisors excluding samples without NNL	52	3.13	22.94	10.92	4.90
Incisors from pregnancies and births in Australia only					
First born children	40	0.00	22.94	9.58	6.26
First born children excluding samples without NNL	33	4.18	22.94	11.61	4.84
First and second born children	39	0.00	22.94	10.36	6.01
First and second born children excluding samples without NNL	35	4.18	22.94	11.54	5.13
First and third bone children	29	0.00	22.94	10.21	6.21
First and third bone children excluding samples without NNL	25	4.18	22.94	11.84	4.99

Table 3. Data for mother and baby characteristics, as well as the neonatal line presence in the donated teeth. Please note that there were 53 mothers interviewed, but the "Total n" column is listing sample size per tooth from each child.

Variable	**Sub-group n**	**Sub-group n**	**Sub-group n**	**Total n**
Number birth	First born n = 50 (77%)	Second born n = 14 (21.5%)	Third born n =1 (1.5%)	65
Past miscarriage	None n = 39 (70%)	One n = 17 (30%)		56
Alcohol	None = 52 (84%)	Occasional n = 10 (16%)		62
Delivery method	Natural n = 36 (55%)	Other n = 14 (22%)	Caesarean n = 15 (23%)	65
Induction	Yes n = 18 (31%)	No n = 41 (69%)		59
Sex of infant	Girl n = 38 (58%)	Boy n = 27 (42%)		65
Stress	Yes n = 24 (37%)	No n = 41 (63%)		65
Illness	Yes n = 29 (45%)	No n= 36 (55%)		65
Neonatal line	Present n = 58 (89%)	Not visible n = 7 (11%)		65

Table 4. Results from the inferential analysis comparing neonatal line thickness between different measures of maternal and baby health. Please note that some mothers donated teeth for more than one child (up to three children). In order to avoid including siblings (i.e. related samples) in the overall analysis, each set of tests was repeated always including only one child from mothers with multiple children. Results column: t = *t*-test, *U* = Mann Whitney *U* test, *H* = Kruskal-Wallis *H* test.

NNL comparisons	**Results**		
Entire dataset n = 65 teeth	***n***	***t/ U/ H***	***p***
Birth number (one, two)	64	*U* = 310.500	0.521
Past miscarriage	65	*H* = 3.264	0.196
Alcohol consumption	62	*U* = 334.500	0.154
Illness	65	*U* = 572.500	0.505
Stress	65	*U* = 401.000	0.216
Delivery method	65	*H* = 3.547	0.170
Induction	59	*U* = 400.000	0.610
Sex of infant	65	*t* = 0.565	0.574
Incisors only, Australia pregnancy and birth only, one tooth per mother max n = 40 (first born child tooth included)			
Past miscarriage	35	*U* = 100.000	0.268
Alcohol consumption (all incisors)	**39**	***U* = 200.000**	**0.001**
Excluding samples without NNL	**32**	***U* = 151.000**	**0.002**
Illness	40	*U* = 202.000	0.795
Stress	40	*U* = 159.000	0.528
Delivery method	40	*H* = 4.146	0.126
Induction	36	*U* = 161.000	0.585
Sex of infant	40	*U* = 171.000	0.452
Past miscarriage	35	*U* = 100.000	0.268
Incisors only, Australia pregnancy and birth only one tooth per mother (excluding related samples) max n = 39 (second born child tooth included)			
Past miscarriage	34	*U* = 111.500	0.381
Alcohol consumption	38	*U* = 138.500	0.265
Illness	39	*U* = 182.000	0.966
Stress	39	*U* = 132.000	0.281
Delivery method	39	*H* = 3.411	0.182
Induction	35	*U* = 151.000	0.358
Sex of infant	39	*U* = 150.500	0.343
Incisors only, Australia pregnancy and birth only, one tooth per mother (excluding related samples) max n = 29 (third born child tooth included)			
Past miscarriage	26	*U* = 64.000	0.525
Alcohol consumption	**28**	***U* = 98.000**	**0.013**
Excluding samples without NNL	**24**	***U* = 78.000**	**0.030**
Illness	29	*U* = 93.500	0.711
Stress	29	*U* = 66.000	0.274
Delivery method	29	*H* = 2.503	0.286
Induction	26	*U* = 99.000	0.241
Sex of infant	29	*U* = 84.500	0.377

Supplement Table 1. Results from the inferential analysis correlating NNL thickness and different maternal and baby characteristics.

Test		**n**			***r***			***p***		
Pearson's (n > 30/ normally distributed)/ Spearman's* (n < 30/ no normal distribution) **Entire dataset**										
NNL thickness correlated with:	Mother's height	64			-0.006			0.962		
	Maternal age at pregnancy	65			-0.211			0.091		
	Number pregnancy	65			-0.50			0.693		
	Maternal age at birth	65			-0.165			0.188		
	Gestation*	65			0.074			0.557		
	Labour length*	56			0.044			0.749		
	Birth weight*	65			0.053			0.677		
	Birth height*	57			-0.197			0.141		
Incisors only, Australia pregnancy and birth only, **accounting for siblings**		1st child tooth	2nd child tooth	3rd child tooth	1st child tooth	2nd child tooth	3rd child tooth	1st child tooth	2nd child tooth	3rd child tooth
NNL thickness correlated with:	Mother's height	39	38	28	-0.070	0.022	-0.057	0.671	0.894	0.773
	Number pregnancy	40	39	29	-0.238	-0.163	-0.321	0.138	0.321	0.090
	Maternal age at birth	40	39	29	-0.284	-0.170	-0.295	0.075	0.302	0.121
	Gestation*	40	39	29	0.118	0.137	0.026	0.470	0.406	0.893
	Labour length*	34	33	25	-0.029	0.070	0.085	0.871	0.699	0.688
	Birth weight*	40	39	29	0.279	0.148	0.304	0.081	0.369	0.108
	Birth height*	35	34	24	-0.176	-0.206	-0.192	0.311	0.242	0.368