Development of the Human Fetal Kidney from Mid to Late Gestation in Male and Female Infants

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Abstract

Background: During normal human kidney development, nephrogenesis (the formation of nephrons) is complete by term birth, with the majority of nephrons formed late in gestation. The aim of this study was to morphologically examine nephrogenesis in fetal human kidneys from 20 to 41 weeks of gestation.

Methods: Kidney samples were obtained at autopsy from 71 infants that died acutely in utero or within 24 h after birth. Using image analysis, nephrogenic zone width, the number of glomerular generations, renal corpuscle cross-sectional area and the cellular composition of glomeruli were examined. Kidneys from female and male infants were analysed separately.

Findings: The number of glomerular generations formed within the fetal kidneys was directly proportional to gestational age, body weight and kidney weight, with variability between individuals in the ultimate number of generations (8 to 12) and in the timing of the cessation of nephrogenesis (still ongoing at 37 weeks gestation in one infant). There was a slight but significant ($r^2 = 0.30, P = 0.001$) increase in renal corpuscle cross-sectional area from mid gestation to term in females, but this was not evident in males. The proportions of podocytes, endothelial and non-epithelial cells within mature glomeruli were stable throughout gestation.

Interpretation: These findings highlight spatial and temporal variability in nephrogenesis in the developing human kidney, whereas the relative cellular composition of glomeruli does not appear to be influenced by gestational age.

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1. Introduction

Nephrogenesis (the formation of nephrons) commences in early development and is complete by birth in the term born human infant (Cullen-McCwenn et al., 2016), with the majority of nephrons (approximately 60%) formed in the second half of gestation (Hinchliffe et al., 1991). Studies of autopsied kidneys have shown that there is a wide range in the number of nephrons in the normal human kidney, from approximately 250,000 to over 2 million (Puelles et al., 2011). The mechanisms leading to such a wide variation in nephron number are currently unknown, but genetic variability, differences in the in utero environment, the rate of nephron loss with aging, as well as exposure to postnatal renal insults throughout life are likely key factors (Cosgrove and Goodyer, 2016; Hoy et al., 2005; Puelles et al., 2011). Since loss of glomeruli ultimately leads to renal disease (Hoy et al., 2005), it is likely that individuals born with a high nephron endowment will be relatively protected from renal disease later in life, whereas individuals born with a low nephron endowment are likely to be more vulnerable. Hence, in order to preserve long-term renal health it is imperative to maximize nephron endowment at birth. In order to develop strategies to do this, it is essential to first develop an

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understanding of normal fetal development, and how factors during pregnancy influence nephron formation.

Over recent years valuable knowledge relating to the regulation of nephrogenesis has been derived from animal models (predominantly rodents) (Takasato and Little, 2015); however, whether these findings can be fully extrapolated to the developing human infant is equivocal (nephrogenic zone width) was assessed (Potter and Thierstein, 1943). However, there were a number of confounding factors in that study relating to in utero growth, exposure to intrauterine inflammation, preterm birth (some infants lived for two to 69 days after birth), and cause of death. Furthermore, only one parameter of kidney growth (nephrogenic zone width) was assessed (Potter and Thierstein, 1943). Importantly, no studies to date have compared nephron development throughout gestation between male and female fetuses.

Therefore, to further enhance our knowledge of the normal development of the human kidney we conducted a comprehensive histological examination of kidney growth from 20 weeks in gestation until term, the developmental period when the majority of nephrons are formed. The aims were to examine: nephrogenic zone width, the number of glomerular generations, glomerular size, and the proportions of different cell types within glomeruli, as well as to explore variability in the timing of the cessation of nephrogenesis. The kidneys from male and female infants were analysed separately, and only the kidneys from infants that were normally grown in utero and died acutely were analysed.

2. Materials and Methods

2.1. Study Groups

In this retrospective study, archival fetal and newborn kidney tissue was obtained from the Women’s and Children’s Hospital in North Adelaide, South Australia, and the Canberra Hospital in the Australian Capital Territory. The kidneys were collected at autopsy from 71 appropriately grown infants who died suddenly in utero or within 24 h after birth. Sixty eight (95.8%) of the infants were born of caucasian mothers, 1 (1.4%) of a Sri Lankan mother, 1 (1.4%) of a Vietnamese mother, and 1 infant (1.4%) was born of an Indigenous Australian mother. The causes of death included asphyxia/cord entanglement (14/71 [20%]), placental abruption (12/71 [17%]), placental infarction/placental thrombosis (8/71 [11%]), extreme prematurity/ respiratory failure (6/71 [8%]), and termination of pregnancy (2/71 [3%]). In 29 of the cases (41%) the cause of sudden death was not determined at autopsy. Five of the infants (7.0%) were exposed to preeclampsia, and 1 infant (1.4%) was exposed to antenatal corticosteroids; in 2 cases (2.8%) the mothers were induced to deliver with prostaglandin treatment. The infants ranged in age from 20 to 41 weeks of gestation (n = 33 female; n = 38 male), whereby gestational age was primarily defined according to early ultrasounds and the date of the mother’s last menstrual period. Autopsies were performed between the years 1996 and 2013, and written informed consent from the parents was obtained for autopsy and archival of tissue. Infants were excluded from the study if there was evidence of congenital abnormalities, intrauterine growth restriction, cardiovascular or renal complications, or exposure to chorioamnionitis or diabetes in utero. Kidneys were also excluded if they were severely macerated. Ethical approval for this study was obtained from the Children, Youth and Women’s Health Service Research Ethics Committee of South Australia and the Australian Capital Territory Human Research Ethics Committee.

2.2. Tissue Preparation and Processing

Kidneys collected at autopsy were weighed and cut into two in the longitudinal plane; large kidneys were further cut transversely. The kidneys were embedded in paraffin and sectioned at 5 μm. Sections were stained with haematoxylin and eosin for the assessment of nephrogenic zone width, number of glomerular generations and renal corpuscle area. Analyses of glomerular cell types were undertaken in a subset of the archived kidneys by immunofluorescence; this was restricted to kidneys where immunofluorescent staining was successful.

2.3. Assessment of Nephrogenesis

In all kidneys it was noted whether nephrogenesis was ongoing or complete. Nephrogenesis was considered ongoing if there was evidence of metanephric mesenchyme and immature nephrons in the form of comma and S-shaped bodies in the outer renal cortex.

2.4. Assessment of Nephrogenic Zone Width and Glomerular Generation Number

In kidneys where nephrogenesis was ongoing, the width of the nephrogenic zone was measured in four randomly sampled regions of the cortex using image analysis software (Image Pro Plus v. 6-0 for Windows, Media Cybernetics; Silver Spring, MD, USA), and the average width per kidney was determined (Gubhaju et al., 2009; Sutherland et al., 2011).

The number of glomerular generations formed within all kidneys was assessed using a medullary ray glomerular counting method (Hinchliffe et al., 1991; Faa et al., 2010; Sutherland et al., 2011). Mature glomeruli were counted along five clearly defined medullary rays per kidney, and the average number of generations per kidney was determined.

2.5. Assessment of Glomerular Size

Glomerular size was assessed by measuring the cross-sectional area of renal corpuscles. To do this, kidney sections were systematically sampled throughout the cortex (inner, middle and outer cortex) at 400 x magnification with a step length of 1 mm. At each field of view, one glomerulus was measured using image analysis software (Image Pro Plus v. 6-0 for Windows, Media Cybernetics; Silver Spring, MD, USA). If more than one was observed in the field of view, the renal corpuscle for analysis was selected according to the method of Nyengaard and Marcussen (Nyengaard and Marcussen, 1993). At least 100 renal corpuscles were measured per kidney, and the average was then calculated.

2.6. Immunofluorescent Identification of Glomerular Cell Types

A standard immunofluorescence protocol (Puelles et al., 2014; Puelles et al., 2015) was used to identify podocytes, endothelial and non-epithelial cells within mature glomeruli; glomeruli in stages I, II and III of development were analysed (Naruse et al., 2000; Sutherland et al., 2011). Briefly, sections were first subjected to heat-induced antigen retrieval in sodium citrate buffer. Immunofluorescent staining was then performed using a DAKO Autostainer Plus Staining System. Wilms’ Tumour-1 (WT-1) antibody (monoclonal mouse M356101, Dako; 1:50, (RRID:AB_564063)) with Alexa Fluor 488 conjugated secondary antibody (A-11001, Invitrogen; 1:2000) was used for the identification of podocytes (previously shown to stain podocyte cytoplasm and foot processes) (Puelles et al., 2014, Puelles et al., 2015), and von Willebrand Factor (vWF) antibody (polyclonal rabbit A006202, Dako);
1:200, (RRID:AB_564062)) with Alexa-Fluor 555 conjugated secondary antibody (A-21428, Invitrogen, 1:1000), was used for the identification of endothelial cells. Nuclei were labelled with 4′,6-diamidino-2-phenylindole (DAPI; D9542-10M6, Sigma–Aldrich; 1:10,000).

2.7. Assessment of the Proportion of Podocytes, Endothelial and Non-epithelial Cells per Glomerulus

Immunofluorescent staining was successful in 14 kidneys (n = 8 male, n = 6 female). Sub-standard immunofluorescence in the remaining kidneys was likely due to inadequate fixation because of the lag time between death and autopsy.

Sections were scanned at 400× using an Aperio ScanScope AT Turbo (Leica Biosystems; Vista, CA, USA). Using Image J (v.6·2, National Institutes of Health; MD, USA), composite images of 50 glomerular cross-sections per kidney (approximately 16 from each of the outer, middle and inner cortex) were assessed. Initially, the total number of DAPI-stained nuclei per glomerulus were counted. Subsequently, WT-1+ podocytes and vWF+ endothelial cells were identified and counted. Cells not labelled as podocytes or endothelial cells in the composite image were classified as non-epithelial cells (the vast majority of these cells being mesangial cells). The relative proportions of each cell type was then calculated, and the average determined per kidney.

2.8. Statistical Analysis

Data were analysed using GraphPad Prism v5·03 for Windows (GraphPad Software Inc.; San Diego, CA, USA). Linear regression analyses were undertaken to determine correlations between gestational age and indices of fetal growth and renal morphology. Male and female infants were examined separately, with an analysis of co-variance performed to compare the regression lines. Initial glomerular generation number in infants with completed nephrogenesis, and renal corpuscle size in 23 week and term infants, was compared between sexes using unpaired two-tailed Student’s t-tests. Values are mean ± standard error of the mean, unless indicated otherwise. Statistical significance was accepted as P < 0·05.

3. Results

3.1. Body and Kidney Weight Gain

Given that body weight is affected by the period of time between delivery and autopsy, we have presented the body weights at the time of birth which were available for the majority of infants (n = 56 (79%); 26 females and 30 males); kidney weights were available for all 71 infants. As expected, in both male and female infants from 20 weeks of gestation until term there was a strong positive correlation between gestational age and body weight (males: r² = 0·92, P < 0·0001; females: r² = 0·92, P < 0·0001) and kidney weight (males: r² = 0·75, P < 0·0001; females: r² = 0·83, P < 0·0001). Female infants were significantly lighter than male infants overall (P = 0·003), with significantly lighter kidneys (P = 0·02); however, the rate of body and kidney weight gain over the gestational period was not different between sexes. Throughout gestation, kidney weight was directly proportional to body weight in both females and males (kidney weight to body weight ratio, males: r² = 9·4 × 10⁻⁸, P = 0·99; females: r² = 0·004, P = 0·73).

3.2. Nephrogenic Zone Width

The morphology of the renal cortex in male and female infants throughout gestation is shown in Fig. 1, and an image depicting the measurement of the nephrogenic zone is shown in Fig. 2a. In both female and male infants, there was a significant inverse correlation between nephrogenic zone width and gestational age (Fig. 2b). There was no difference between sexes in overall nephrogenic zone width, or the rate of change in nephrogenic zone width over the gestational period (Fig. 2b).

3.3. Timing of the Cessation of Nephrogenesis

Table 1 indicates whether nephrogenesis was ongoing or had ceased at the time of autopsy in all kidneys studied. All infants ranging in age from 20 to 31 weeks of gestation had ongoing nephrogenesis, whereas it was complete in all term infants at 38–41 weeks of gestation. Between 35 and 37 weeks of gestation, there was variability in the timing of the cessation of nephrogenesis (Table 1). Fig. 3 shows kidneys from two...
infants at the same gestational age (37 weeks gestation) with nephrogenesis ongoing in one of the kidneys and complete in the other.

3.4. Number of Glomerular Generations

Measurement of glomerular generation number in the fetal kidneys is depicted in Fig. 4a and b. There was a strong positive relationship between the number of glomerular generations and gestational age (Fig. 4c), kidney weight (Fig. 4d) and body weight (Fig. 4e), in both female and male infants. There was no significant difference between sexes in the number of glomerular generations formed, or the rate of formation over the gestational period (Fig. 4c).

In kidneys in which nephrogenesis was complete, the average number of glomerular generations was not significantly different between sexes (female: \(9.9 \pm 0.23\), male: \(10.4 \pm 0.29\); \(P = 0.22\)); overall, however, the final number of glomerular generations was variable between infants, ranging from 8 to 12 per kidney. For example, in some kidneys examined where nephrogenesis was ongoing at 33 weeks of gestation, there were 9–10 glomerular generations (Fig. 4a); whereas, in other kidneys where nephrogenesis was complete at 35 weeks of gestation there were 8–9 glomerular generations.

3.5. Renal Corpuscle Cross-Sectional Area

In females there was a small but significant positive correlation between renal corpuscle cross-sectional area (depicted in Fig. 5a–d) and gestational age, increasing from an average of \(3631 \pm 594\ \mu m^2\) at 23 weeks of gestation to \(5136 \pm 235\ \mu m^2\) at term (\(P = 0.02\)); in males, however, this relationship was not evident (23 weeks gestation: \(4677 \pm 238\ \mu m^2\), term: \(5021 \pm 158\ \mu m^2\); \(P = 0.24\) (Fig. 5e)). Similarly, females (but not males) showed a significant positive correlation between renal corpuscle cross-sectional area and kidney weight (Fig. 5f), body weight (Fig. 5g), and the number of glomerular generations formed within the cortex (Fig. 5h). In infants at 23 weeks gestation (\(P = 0.13\); \(n = 3\) females and 4 males), and at term (\(P = 0.67\); \(n = 12\) females and 13 males), there was no statistically significant difference in average renal corpuscle cross-sectional area between the sexes.

3.6. Glomerular Cell Types

Representative images of immunofluorescently labelled glomeruli are shown in Fig. 6a–c. The average numbers of podocytes, endothelial cells and non-epithelial (mesangial) cells per fully-formed glomerulus in cross-section are shown in Fig. 6d (female infants; \(n = 6\)) and Fig. 6e (male infants; \(n = 8\)). The average number of each cell type remained constant across gestation, with no apparent relationship between gestational age and the number of podocytes, endothelial cells or non-epithelial cells (Fig. 6). Overall, the average proportion of podocytes within glomeruli was \(64.4 \pm 0.9\%\); the proportion of endothelial cells was \(27.6 \pm 1.0\%\), and the proportion of non-epithelial cells was \(8.0 \pm 0.5\%\); the proportions of the glomerular cell types were not correlated with gestational age (Fig. 6). It is expected that the majority of the non-epithelial cells would be resident mesangial cells; however, this could not be verified.

4. Discussion

This comprehensive study of human fetal kidneys shows that kidney growth in the latter half of gestation is directly proportional to body weight and gestational age in both male and female infants. There were some sex differences in renal growth, with only female infants exhibiting a significant increase in glomerular size (in fully developed
glomeruli) from mid gestation to term. Importantly, our findings also highlight that there is a variation in the timing of the cessation of nephrogenesis, and in the final number of glomerular generations formed within human kidneys, which likely influences nephron endowment.

Relatively few studies to date have examined nephrogenesis during late gestation in humans (Hinchliffe et al., 1991; Faa et al., 2010; Sutherland et al., 2011; Chikkaniaiah et al., 2012; Potter and Thierstein, 1943; dos Santos et al., 2006; Fonseca Ferraz et al., 2008; Souster and Emery, 1980; Crobe et al., 2014; Hinchliffe et al., 1992). The majority of previous studies have analysed only a small cohort of infants, and many (but not all) were confounded by the inclusion of infants that were growth restricted prior to birth. This is an important limitation because it is well-established in animal models that intrauterine growth restriction adversely impacts nephrogenesis leading to a reduction in nephron endowment (Zohdi et al., 2012). Hence, in the present study we have ensured that any infants that were intrauterine growth restricted or exposed to chorioamnionitis (which can also adversely impact nephrogenesis (Galinsky et al., 2011)) were excluded from the study. Importantly, we were also careful to only analyse kidneys that were obtained from infants that had been growing normally in utero and died suddenly; this information was derived from autopsy reports. Using this approach, we eliminated infants that may have been in poor health over a chronic period in utero, and therefore the infants selected for the study represent, as closely as possible, normal intrauterine renal development. Furthermore, we compared indices of renal growth between male and female fetuses throughout gestation; to our knowledge this analysis has not been conducted in any prior study.

4.1. Males are Larger than Age-Matched Females with Kidney Weight Proportional to Body Weight

As expected, infant body weight and kidney weight were both strongly positively correlated with gestational age in both female and male infants. As a result, the kidney weight to body weight ratio remained constant over the gestational period. Similar findings in relation to fetal kidney weight at autopsy (Mitropoulos et al., 1992) have been previously reported. Female infants were significantly lighter in weight overall, with significantly reduced kidney weight in comparison to males. Our findings are consistent with previous studies demonstrating that male infants in late gestation are significantly heavier at birth than females at the same gestational age, and that kidney size during development (length and volume) is less in females compared to males (Sampaio, 1992; Verburg et al., 2007). Notably, our findings show that the growth of the kidneys (weight gain over time) in the second half of gestation is not different between the sexes.

There was variation in the timing of the cessation of nephrogenesis and in the number of glomerular generations formed. As expected, and consistent with previous studies (dos Santos et al., 2006; Fonseca Ferraz et al., 2008; Sutherland et al., 2011), there was a strong inverse correlation between the width of the nephrogenic zone and gestational age. In early studies, Potter et al. 1943 (Potter and Thierstein, 1943) demonstrated a link between the timing of the cessation of nephrogenesis and infant body size (body weight and length), which would also likely relate to gestational age. However, the findings of that study are somewhat difficult to interpret given no discrimination was made between the fetuses and infants studied in relation to body growth in utero, exposure to intrauterine inflammation, length of postnatal survival, or cause of death.

Nephrogenesis is often quoted in the literature to be completed late in gestation at around 36 weeks of gestation. Although our findings are consistent with this, there appears to be some variation in the cessation in the timing of nephrogenesis; in a previous study we have observed nephrogenesis to be complete as early as 32 weeks of gestation (Sutherland et al., 2011) and in this study there was evidence of ongoing nephrogenesis at 37 weeks of gestation.

In the present study we were unable to directly determine the total number of glomeruli/nephrons within the kidneys as it was not known what fraction of the whole kidney was collected at autopsy. As a surrogate indicator, we examined the number of glomerular generations present within the renal cortex, which is known to correlate with nephron number (Hinchliffe et al., 1992). We found that there was wide variation between individuals in the total number of generations formed, ranging from eight to twelve per kidney, which is relatively consistent with previous studies (Faa et al., 2010; Sutherland et al., 2011; dos Santos et al., 2006). This variation in the number of glomerular generations formed within the kidneys of developing infants is likely to be a major contributor to the wide range in nephron endowment observed in human subjects. In this regard, how final nephron number is determined in the mammalian kidney is not known (Kopan et al., 2014). In rodents, where nephron formation naturally continues into the immediate postnatal period (Rumballe et al., 2011), cessation of this process can potentially be explained by either an extrinsic environmental trigger associated with parturition, or an intrinsic clock which triggers differentiation (Hartman et al., 2007; Rumballe et al., 2011). Recent studies in the mouse showed a clear distinction in gene expression and proliferation rates between nephron progenitors isolated early versus late in gestation, supporting an intrinsic model of aging (Chen et al., 2015). In humans a parturition-based trigger appears unlikely, given...
that nephron formation is complete some weeks before birth. It may, however, relate to the late gestational cortisol surge (Liggins, 1994) which plays a key role in lung maturation prior to birth (Murphy, 1978). Indeed, exposure to exogenous antenatal steroids has been shown to accelerate nephrogenesis in preterm born infants (Gubhaju et al., 2009), which supports this concept.

In future studies, it is imperative to develop an understanding of what controls the pace and number of glomerular generations formed within the human kidney, in order to evaluate risk and develop strategies to maximize nephron endowment at birth, which will in turn, favourably impact lifelong renal health.

4.2. Differences Evident in the Growth of Glomeruli between the Sexes

In this study, only fully-formed mature glomeruli were included in our analyses of glomerular size and cellular composition. An interesting finding of our study was the apparent sexual dimorphism in the growth of glomeruli during late gestation. In females there was a positive correlation between renal corpuscle cross-sectional area and gestational age, with average renal corpuscle cross-sectional area increasing from 3631 ± 594 μm² at 23 weeks of gestation to 5136 ± 235 μm² at term. To the contrary, in males renal corpuscle size remained relatively constant over the gestational period, averaging 4677 ± 238 μm² at 23 weeks of gestation.
gestation and 5021 ± 158 μm² at term. One previous study by Fonseca Ferraz et al. (2008) in 86 non-growth restricted fetuses, found that the size of mid-cortical glomeruli significantly increased with increasing gestational age, whereas the size of outer and inner cortical glomeruli remained constant. It is difficult, however, to compare our findings with this previous study given that we have systematically sampled the kidneys (and not differentiated glomeruli according to location within the cortex), and because males and females were not assessed separately in the previous study.

Why there appears to be sexual dimorphism in glomerular growth in the second half of gestation is currently unknown. However, this is an interesting finding which warrants further investigation. Besides gestational age, renal corpuscle size in females also significantly increased along with increasing body weight, kidney weight, and glomerular generation number (each of these also a correlate of gestational age), so it is unknown which factor(s) may be underlying the increased rate of renal corpuscle growth observed in the female infants.

Renal corpuscle size in term infants was not significantly different between sexes, however one previous study has shown that in children (0–15 years of age) the size of the renal corporules in the outer and middle regions of the cortex are significantly larger in females compared to males (Moore et al., 1993). It may be speculated that this enlargement relates to a lower nephron endowment in the female kidneys, resulting in compensatory hypertrophy of the glomeruli as the children grow. However, in this study we did not observe any differences in glomerular generation (nephron) number between sexes. Furthermore, it is very unlikely that this mechanism explains the increasing rate of growth we have observed during gestation, as the fetal kidneys have very few functional requirements in utero, and the observed increase in renal corpuscle size in females was positively correlated with glomerular generation number.

4.3. Proportion of Podocytes within Glomeruli remains Constant

Podocytes play a critical role in glomerular filtration, with the depletion of podocytes linked to the development of renal disease (Puelles and Bertram, 2015). An important aspect of our study, therefore, was to examine the cellular composition of glomeruli over the gestational period. Unfortunately, we were restricted in the number of kidneys we could analyse by whether the immunostaining was successful; this was likely dependent on the time between death and tissue fixation at autopsy.

Importantly, our findings suggest that once a glomerulus has formed and is in a mature stage of development, whether it is early in gestation or late in gestation, the number and proportion of podocytes relative to other glomerular cell types appears to remain relatively constant; this was applicable to the kidneys of both male and female infants. These findings are in contrast to those of Crobe et al. (2014), who reported that podocyte number in glomerular cross-sections decreases with increasing gestational age. It is important to note, however, that there were a number of confounders in that study, with the inclusion of both intrauterine growth restricted and preterm infants, as well as limitations regarding the use of a non-specific staining method to identify podocytes. Notably, in a recent study where the absolute number of podocytes per glomerulus were counted using stereological techniques it was shown that there is about a 1.8 fold increase in the number of podocytes formed in glomeruli as they progress from an immature form (pre-capillary loop stage) to the capillary loop stage form.
In our study only mature glomeruli were examined, however a limitation of our study is that cell numbers were assessed in glomerular cross-sections, and so the absolute number of podocytes per glomerulus was not determined.

5. Conclusion

The findings of this study highlight the considerable variation in the number of glomerular generations formed within the human kidney during gestation, as well as differences in the timing of the cessation of nephrogenesis. It is imperative to now elucidate the mechanisms leading to this spatial and temporal variability in renal development, as both have the potential to influence total nephron endowment. Furthermore, our findings suggest that late gestational glomerular growth differs in male and female infants, which is also an important avenue for future research.

Conflicts of Interest

The authors have no conflicts of interest relevant to this article to disclose.

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Author Contributions

MJB conceived the study, with contributions from all authors. Archived tissue and autopsy reports were provided by ALK, JED, and LM. DR and TJF performed the experimental analysis, with data analysis performed by DR. DR prepared the manuscript, with editorial assistance from all other authors. All authors have reviewed and approved the final manuscript.

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