Received: 11 January 2022 | Revised: 8 April 2022 | Accepted: 15 April 2022

DOI: 10.1002/ajmg.a.62764

RAPID COMMUNICATION

AMERICAN JOURNAL OF medical genetics PART A WILEY

NAD+ deficiency in human congenital malformations and miscarriage: A new model of pleiotropy

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Abstract

Pleiotropy is defined as the phenomenon of a single gene locus influencing two or more distinct phenotypic traits. However, nicotinamide adenine dinucleotide (NAD+) deficiency through diet alone can cause multiple or single malformations in mice. Additionally, humans with decreased NAD+ production due to changes in pathway genes display similar malformations. Here, I hypothesize NAD+ deficiency as a pleiotropic mechanism for multiple malformation conditions, including limb-body wall complex (LBWC), pentalogy of Cantrell (POC), omphalocele-exstrophy-imperforate anus-spinal defects (OEIS) complex, vertebral-anal-cardiac-tracheoesophageal fistula-renal-limb (VACTERL) association (hereafter VACTERL), oculoauriculovertebral spectrum (OAVS), Mullerian duct aplasia-renal anomalies-cervicothoracic somite dysplasia (MURCS), sirenomelia, and urorectal septum malformation (URSM) sequence, along with miscarriages and other forms of congenital malformation. The term Congenital NAD Deficiency Disorder (CNDD) could be considered for patients with these malformations; however, it is important to emphasize there have been no confirmatory experimental studies in humans to prove this hypothesis. In addition, these multiple malformation conditions should not be considered individual entities for the following reasons: First, there is no uniform consensus of clinical diagnostic criteria and all of them fail to capture cases with partial expression of the phenotype. Second, reports of individuals consistently show overlapping features with other reported conditions in this group. Finally, what is currently defined as VACTERL is what I would refer to as a default label when more striking features such as body wall defects, caudal dysgenesis, or cloacal exstrophy are not present.

KEYWORDS
diabetic embryopathy, discordant twins, LBWC, NAD+, OEIS, VACTERL

1 | PART I: EVIDENCE THAT MULTIPLE MALFORMATION CONDITIONS ARE NOT DISTINCT ENTITIES: INTRODUCTION

Despite advances in genetic testing, including chromosomal microarray, exome sequencing, genome sequencing, and newer approaches including transcriptome and methylation analyses, there remains a group of conditions which include anomalies originating in early embryonic development for which a mechanism, genetic or otherwise, has yet to be identified (Innes & Lynch, 2021). In an effort to generate study of these conditions, some members of this group were labeled Recurrent Constellations of Embryonic Malformations (RCEM) including pentalogy of Cantrell (POC), limb-body wall complex (LBWC), omphalocele-exstrophy-imperforate anus-spinal defects (OEIS) complex, vertebral-anal-cardiac-tracheoesophageal fistula-renal-limb (VACTERL), oculoauriculovertebral spectrum (OAVS), and Mullerian duct aplasia-renal anomalies-cervicothoracic somite dysplasia (MURCS; Adam et al., 2020). Additional conditions with multiple malformations for which no causative mechanism has been elucidated include sirenomelia and urorectal septal malformation (USRM) sequence. Since 1836, when sirenomelia was perhaps first described in modern medicine by Saint-Hilaire (Boer et al., 2017), these patterns of malformations have been divided into reportedly discrete conditions, with attempts to delineate specific diagnostic criteria, in hopes of finding a unique pathogenic mechanism for each. However, this technique of splitting these conditions has been largely unsuccessful in identifying a pathogenic mechanism except for identifying variants in *CHD7* for CHARGE association (now syndrome; Vissers et al., 2004). Here, evidence is presented to show these described conditions are not discrete entities with specific criteria separating them from each other but are part of a single, large entity.

1.1 | There is no uniform consensus of clinical diagnostic criteria for each of these conditions and all of them fail to capture cases with partial expression of the phenotype

VACTERL, the multiple malformation condition with by far the most reported patients, also has the greatest number of individuals who do not meet reported criteria, partly because the criteria themselves are not agreed upon. In a survey of 121 clinical geneticists, 15% stated at least two features were necessary to meet criteria, 79% needed three, and 8% required four criteria to be classified as having VACTERL (Solomon et al., 2012). A different study stated there must be the presence of three or more features including at least one major feature, defined as tracheoesophageal fistula with or without esophageal atresia (TEF ± EA), anal atresia, or either a radial ray defect or postaxial polydactyly (Adam et al., 2020). Minor features included structural cardiac malformations, vertebral segmentation anomalies, and renal hypoplasia/aplasia. The EUROCAT VACTERL Study defined STRICT-VACTERL as cases with ≥3 major VACTERL features and no major congenital anomalies outside the VACTERL spectrum (with specific restrictions mentioned; van de Putte, van Rooij, Marcelis, et al., 2020). In this evaluation of 501 cases recorded with VACTERL, there were 213 cases which met their specific criteria (43%), but there were 82 cases (16%) defined as VACTERL-LIKE (<3 major VACTERL features) and 102 cases (20%) labeled as VACTERL-PLUS, (having additional findings besides the cardinal features). They excluded an additional 104 patients (21%) who only had two cardinal features and did not meet their criteria for STRICT-VACTERL. Thus, of the 501 cases originally analyzed, less than half met their specific criteria for STRICT-VACTERL. In a different VACTERL study, 82/103 isolated VACTERL cases had <3 major features, leaving only 21 cases (20%) which met the criteria of requiring three or more VACTERL features (Rittler et al., 1996). Adding to the confusion of the acronym and diagnostic criteria, investigators have recently proposed to add spinal dysraphism, resulting in the acronym VACTERLS (Amelot et al., 2020), or adding G for genital anomalies to VACTERL (Forero & Bird, 2021).

There are multiple examples demonstrating lack of diagnostic uniformity and partial expression in other multiple malformation conditions. POC was defined as the presence of the following five criteria: midline supraumbilical abdominal wall defect, defect of the anterior diaphragm, defect of the lower sternum, defect of the diaphragmatic pericardium, and congenital heart defect (Cantrell et al., 1958). However, Toyama stated all five must be present for complete POC, probable (or suspected) POC if four of the five (which must include an intracardiac defect and ventral abdominal wall defect) were present, and incomplete if less than four criteria were met (Toyama, 1972). A different study lists omphalocele as the suggested abdominal defect (Adam et al., 2020), yet Toyama's review of 36 cases lists the following abdominal wall defects: omphalocele, diastasis recti, ventral hernia, absent umbilicus, and eventration. This suggests difficulty in finding uniform criteria for at least one aspect (midline supraumbilical abdominal wall defect) of this condition, demonstrating lack of consensus for the diagnostic criteria of POC.

LBWC has generally been described as the presence of at least two of the following: exencephaly or encephalocele with facial clefts, thoracoschisis, abdominoschisis, or both, and limb defects (Van Allen et al., 1987). Craven, Carey, and Ward presented three cases with no exencephaly/encephalocele, and the only limb defects were contractures and rocker bottom feet, suggesting limb-body wall defect with umbilical cord agenesis as a subgroup (Craven et al., 1997). Martinez-Frias reported 15 cases of body wall complex without limb defects, five of which had either hydrocephaly or cranial disruption by bands, but not true exencephaly or encephalocele (Martinez-Frias, 1997). There have also been cases reported with facial clefts (with or without cranial or central nervous system [CNS] defects) with limb defects, but without body wall defects (Halder, 2010) which some clinicians believe do not meet the criteria for LBWC (no body wall defect).

OEIS complex was originally defined as midline abdominal and pelvic defects of omphalocele, exstrophy of the bladder, imperforate anus, and spine abnormalities (Carey et al., 1978). However, in the original article, 23 of 29 patients detected in a hospital records review had either two or three of the features, but not complete OEIS (Carey et al., 1978). A study of cases in Mexico described the phenotypic

spectrum of the 12 cases, of which four were OEIS, four were OES (omphalocele, exstrophy, and spinal defect), two were EIS (exstrophy, imperforate anus, and spinal defect), and two were OEI, (omphalocele, exstrophy, and imperforate anus; Arteaga-Vázquez et al., 2019).

Urorectal septum malformation (URSM) sequence includes absence of anal and other perineal openings in association with ambiguous genitalia along with urogenital and lower intestinal anomalies (Escobar et al., 1987). Continuing the spectrum, Wheeler and Weaver described 25 cases of partial urorectal septum malformation sequence, using the diagnostic criteria of a single perineal/anal opening that drained a common cloaca in combination with an absent (imperforate) anus, which was thought to improve long-term survival (Wheeler & Weaver, 2001). Additionally, URSM has been considered as part of both OEIS and LBWC (Heyroth-Griffis et al., 2007; Keppler-Noreuil, 2001).

MURCS criteria has been suggested to be Mullerian duct hypoplasia/aplasia with one or more of the following: short, blind ending vagina, renal anomalies including agenesis, hypoplasia, and malposition, or vertebral segmentation anomalies (Adam et al., 2020). There have been rare reports of MURCS with cardiac and digital anomalies, suggesting this might be a variant of what is labeled VACTERL (Guerrier et al., 2006), along with cases in males with azoospermia or thin vas deferens (Meschede et al., 1998; Wellesley & Slaney, 1995). In the original report of 30 patients by Duncan et al. (1979) there were 11 cases with rib anomalies and eight cases with upper extremity malformations, suggesting some patients may either not meet the criteria or have additional features not recognized by the criteria.

1.2 | Reports of cases consistently show overlapping features with other conditions in this group

Historically, there have been attempts to think of some of these conditions as part of a spectrum and not individual conditions. For example, sirenomelia was suggested to be on a spectrum with hemifacial microsomia (also called OAVS) and VATER (now called VACTERL; Duncan & Shapiro, 1993; Duncan & Shapiro, 1988). Nontraditional findings in sirenomelia included TEF ± EA, CNS, ear, face, cleft lip and/or palate, and eye anomalies. Nontraditional findings in hemifacial microsomia patients included anal dysgenesis, TEF ± EA, and cardiovascular, pelvic, CNS, abdominal wall, genital, and respiratory anomalies (Duncan & Shapiro, 1993).

In addition to cardiac and abdominal wall anomalies, patients with POC have been described as having defects in the following organ systems: CNS, renal, gastrointestinal (GI), respiratory, skeletal, and limb, in addition to head and neck findings including cleft lip and palate, hypertelorism, and micrognathia (Jnah et al., 2015). LBWC patients have been described to have nontraditional anomalies including renal, cardiac, vertebral, rib, intestinal, bladder, genitalia and cleft lip, and palate (Colpaert et al., 2000; Hunter et al., 2011). URSM sequence patients have been noted to have CNS, renal, TEF, vertebral, limb, and cardiac anomalies (Wheeler & Weaver, 2001).

In the EUROCAT VACTERL Study, the following anomalies were noted in patients: CNS anomalies including anencephaly, encephalocele, hydrocephalus, and spina bifida, sensory organ anomalies including anophthalmos, microphthalmos, and ear malformations causing hearing loss, respiratory system anomalies, digestive system anomalies, urogenitary anomalies, musculoskeletal anomalies, lower limb anomalies, cleft lip and palate, and other anomalies including the skin and lymphatic systems (van de Putte, van Rooij, Marcelis, et al., 2020).

In summary, the following organ systems have been reported to have defects in patients described to have LBWC, POC, OEIS, sirenomelia, OAVS, and VACTERL: cardiovascular, vertebral/ribs, urogenitary, sensory, limbs/digits, palate, CNS, respiratory, gastrointestinal, skin and lymphatic (Arteaga-Vázquez et al., 2019; Aslan et al., 2004; Carey et al., 1978, Colpaert et al., 2000; Duncan & Shapiro, 1993; El Mansoury & Mbekeani, 2016; Fernández et al., 1997; Grigore et al., 2018; Hunter et al., 2011; Jensen et al., 1993; Jnah et al., 2015; Keppler-Noreuil, 2001; Toyama, 1972; van de Putte, van Rooij, Marcelis, et al., 2020).

1.3 | All conditions in this group share overlapping cardinal features with at least one other condition in this entity, and specifically VACTERL overlaps with all of these reported conditions

Previous reports have listed minimum clinical diagnostic criteria in an attempt to describe some of the multiple malformation conditions (Adam et al., 2020; Escobar et al., 1987; Lhuaire et al., 2013). In reviewing these criteria, the following overlapping cardinal features are identified: Body wall defects-LBWC, OEIS, POC, Vertebral-MURCS, OAVS, VACTERL. Cardiac-POC, VACTERL. Renal-sirenomelia, MURCS, VACTERL. Limb-LBWC, sirenomelia, VACTERL. Genitourinary (excluding renal)-MURCS, OEIS, sirenomelia, URSM (Table 1).

The only groups of defects not included in VACTERL are body wall defects (LBWC, OEIS, POC), extreme genitourinary anomalies such as cloacal exstrophy and caudal dysgenesis seen in OEIS, URSM, and sirenomelia, or milder genitourinary anomalies such as those found in MURCS, where genital anomalies are not yet considered part of the VACTERL acronym. What is currently called VACTERL is what I would refer to as a default label when more striking features such as body wall defects or distal defects such as caudal dysgenesis or cloacal exstrophy are not present. This seems logical because vertebral, cardiac, renal and limb anomalies are the most common anomalies associated with multiple malformation conditions. Of note, it is unknown how many children are born with isolated vertebral or renal anomalies, as imaging is not routinely obtained to evaluate the vertebrae and kidneys without another medical reason requiring these studies. Cardiac anomalies are arguably the most common VACTERL defect, with an incidence of roughly 1/100 livebirths (Liu et al., 2019). Limb defects, while not as frequent, are the most visibly obvious of these four common anomalies and can initiate the workup for other VACTERL anomalies when noted. Also, some clinicians consider vertebral, cardiac and renal anomalies so common as to be labeled minor

TABLE 1 Multiple malformation conditions with overlapping anomalies denoted in italicized and bold type

Limb-body wall complex (LBWC)
Presence of at least two of the following:
• Exencephaly or encephalocele with facial clefts
• ***Thoracoschisis, abdominoschisis, or both***
• ***Limb defects***
Mullerian duct aplasia, renal anomalies, cervicothoracic somite dysplasia (MURCS)
Mullerian duct hypoplasia/aplasia with one or more of the following:
• Short, blind vagina
• ***Renal agenesis, hypoplasia, or malposition, including pelvic kidney***
• ***Vertebral segment anomalies***
Oculoauriculovertebral spectrum (OAVS)
Presence of at least two of the following:
• Unilateral microtia or external aural atresia
• Unilateral mandibular hypoplasia
• Unilateral or bilateral epibulbar dermoids
• ***Vertebral segmentation anomalies***
Omphalocele-exstrophy-imperforate anus-spinal defects (OEIS complex)
• ***Omphalocele***
• ***Exstrophy of the cloaca***
• ***Imperforate anus***
Pentalogy of Cantrell (POC)
Presence of at least three of the following:
• ***Omphalocele***
• Defect of the anterior diaphragm
• Defect of the lower sternum
• Defect of the diaphragmatic pericardium
• ***Congenital heart defect(s)***
Sirenomelia
Presence of the following in association with single lower limb:
• ***Unilateral or bilateral renal agenesis***
• ***Absence of external genitalia***
• ***Anorectal atresia***
• Aberrant arterial abdominal vascularization
Urorectal septum malformation (URSM) sequence
• ***Absence of anal and other perineal openings***
• ***Ambiguous genitalia***
• ***Urogenital anomalies***
• Lower intestinal anomalies
Vertebral-anal-cardiac-tracheoesophageal fistula-renal-limb (VACTERL association)
Presence of three or more features including at least one major:
• *Major features*
• Tracheoesophageal fistula with or without esophageal atresia
• ***Anal atresia***
• ***Radial ray defect or postaxial polydactyly***
• *Minor features*
• ***Structural cardiac malformations***
• ***Vertebral segmentation anomalies***
• ***Renal hypoplasia/aplasia***

Note: From Adam et al. (2020), Escobar et al. (1987), and Lhuaire et al. (2013).

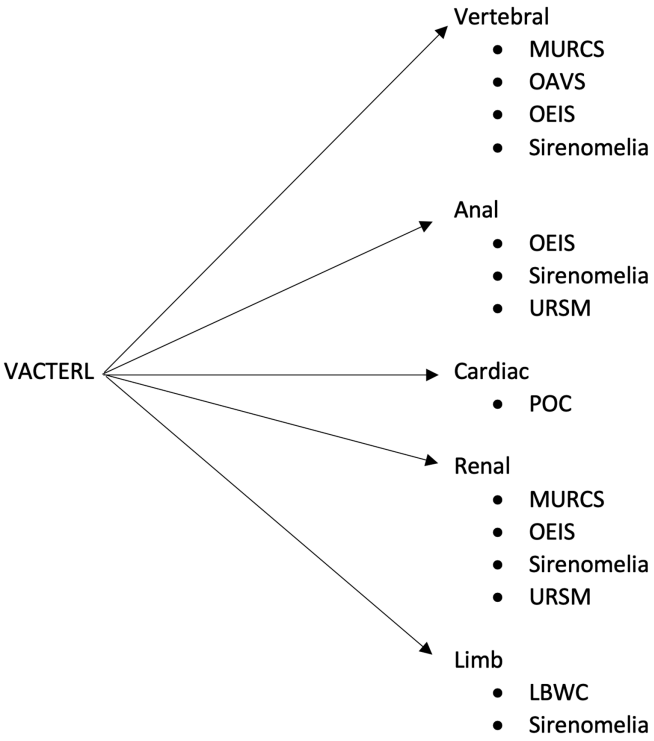

FIGURE 1 Features of VACTERL present in other recurrent multiple malformation conditions. LBWC, limb-body wall complex; MURCS, Mullerian duct aplasia-renal anomalies-cervicothoracic somite dysplasia; OAVS, oculoauriculovertebral spectrum; OEIS, omphalocele-exstrophy-imperforate anus-spinal defects; POC, pentalogy of Cantrell; URSM, urorectal septum malformation; VACTERL, vertebral-anal-cardiac-tracheoesophageal fistula-renal-limb

anomalies and require TEF ± EA, anal atresia, or radial ray defects or postaxial polydactyly to be present to meet the requirements for a VACTERL diagnosis (Solomon et al., 2012; Adam et al., 2020). As mentioned previously, each of the VACTERL anomalies have been described in cases of POC, LBWC, OEIS, sirenomelia, and OAVS. Thus, it is only when the features of other more extreme conditions are absent that the diagnosis of VACTERL is considered (Figure 1).

2 | PART II: EVIDENCE NAD+ DEFICIENCY CAN CAUSE CONGENITAL MALFORMATIONS AND MISCARRIAGE: A CONCISE REVIEW OF NAD+

Nicotinamide adenine dinucleotide (NAD) is found in all parts of cells, including the nucleolus, nucleus, mitochondria, cytoplasm, and

organelles including ribosomes, endoplasmic reticulum, and Golgi apparatus (Koch-Nolte et al., 2011). NAD exists in either its oxidized form (NAD+) or its reduced form (NADH). Under physiologic conditions, the cytosolic NAD+/NADH ratio is ~700, whereas the mitochondrial NAD+/NADH ratio is approximately 7–8 (Williamson et al., 1967; Zhang & Ying, 2019), thus most references emphasize the oxidized form, NAD+. It has two primary functions. The first is its indispensable role as an essential cofactor in cellular oxidation/reduction reactions for production of the primary energy source in human cells, adenosine triphosphate (ATP), through glycolysis in the cytoplasm and oxidative phosphorylation in the mitochondria (Katsyuba et al., 2020). The second is as essential substrate for multiple NAD+ dependent enzyme families including sirtuins, ADP-ribose transferases including Poly (ADP-ribose) polymerases (PARPs), and cyclic ADP (cADP)-ribose synthases (Zapata-Pérez et al., 2021). Briefly, sirtuins are critical for DNA repair and metabolic regulation, and they influence age-related diseases including diabetes and atherosclerosis (Finkel et al., 2009). In addition to their impact on metabolism, PARPs modulate chromatin structure, transcription, replication, recombination, and DNA repair (Morales et al., 2014).

NAD+ is produced by two pathways: one requires the essential amino acid tryptophan, and the other requires dietary niacin (Zapata-Pérez et al., 2021; Figure 2). The NAD+ de novo synthesis pathway catabolizes tryptophan through the kynurenine pathway, and the NAD+ salvage pathway converts niacin and other precursors into NAD+. A failure to produce an adequate amount of NAD+ due to inhibition of either pathway, decreased NAD+ availability due to increased consumption, or disturbances in the NAD^+/NADH ratio in any cellular process can also create serious health ramifications (Katsyuba et al., 2020; Zapata-Pérez et al., 2021). Here, the focus will be on the role of NAD+ during embryonic and fetal development.

2.1 | Phenotypic findings of mice with environment only or gene–environment NAD+ deficiency

In 2020, a remarkable study demonstrated for the first time that malformations could be produced solely by NAD+ deficiency due to environmental factors (dietary restriction of tryptophan and niacin, with or without hypoxia) in wild type mice (all genetically identical; Cuny et al., 2020). Here is a summary of the four seminal findings:

1. Wild type mice with only diet restriction (reduced tryptophan and niacin) caused multiple malformations and embryo loss.
2. Hypoxia increased the likelihood of embryo malformation in wild type mice with diet restrictions.
3. In maternal mice, *Haao* loss of function (LoF) variant exacerbated the effect of dietary restriction on embryonic development.
4. Maternal and embryonic NAD levels (NAD+ and NADH combined) are lowered under maternal treatment conditions (dietary restriction of tryptophan and niacin, with or without hypoxia) that cause embryo loss and congenital malformations of mouse embryos.

Thus, no genetic influence through DNA variants was required to cause these anomalies or embryo losses, emphasizing the importance of NAD+ by itself in embryonic development. Unlike human NAD levels, which have not been studied in pregnancy, the maternal and embryonic NAD levels could be studied in mouse models, demonstrating decreased maternal NAD levels due solely to pregnancy itself, as well as varying degrees of NAD deficiency in the mothers and embryos due to dietary restrictions, hypoxia, and loss-of-function variants in *Haao*. Again, the variant in *Haao* exacerbated the effect of dietary restriction on NAD levels (gene–environment interaction) but was not necessary to cause malformations (Cuny et al., 2020).

The mice in the above study had malformations in the following locations: skull (exencephaly, a neural tube defect), eyes, palate, vertebrae and ribs, heart, abdominal wall, kidneys, limbs, digits, and tail in the form of caudal agenesis (Table 2). It should be noted the mice were not phenotyped for all organ systems. However, given the finding of caudal agenesis, gastrointestinal and reproductive tract anomalies could have occurred. Also, the mice were not carried to term to evaluate for abnormalities usually occurring in the fetal period of development such as brain growth (microcephaly) or short stature.

It is important to emphasize just how variable the phenotypic findings were between individual mice. Despite the mothers being genetically identical and being offered the exact same defined diet, there was inter-litter variability (some litters with all embryos resorbed, some with all mice having anomalies, some with no anomalies, and some with a mixture of these findings), intra-litter variability (some mice with none, one, two or more anomalies, or resorption in the same litter), and individual variability (unique single and multiple anomaly combinations; Figure 3). This demonstrates the incredibly delicate balance of NAD+ requirements necessary for proper embryonic development of all organ systems. For example, just a small increase of nicotinic acid in drinking water (~20%) prevented defects in *Haao* and *Kynu* null mouse embryos (Shi et al., 2017). It is also important to emphasize many mouse embryos had a single abnormality, suggesting a possible mechanism for isolated birth defects in humans, along with the fact that multiple embryos were resorbed, suggesting a mechanism for human miscarriage.

2.2 | Congenital malformations identified in patients with biallelic variants in NAD+ de novo synthesis pathway genes

Although NAD levels in pregnant women and human embryos have not been studied, there is still evidence NAD+ is critical for human embryonic and fetal development. In 2017, the first four patients with confirmed biallelic variants in the NAD+ de novo synthesis pathway were reported (Shi et al., 2017). Since that time, an additional 18 reported or known individuals with biallelic variants in *HAAO*, *KYNU*, or *NADSYN1* have been described (Szot et al., 2020; Ehmke et al., 2020; L. Bird, personal communication, September 14, 2021; Schüle et al., 2021; Szot et al., 2021). Initially these patients were labeled as having Vertebral, Cardiac, Renal and Limb Defects (VCRL)

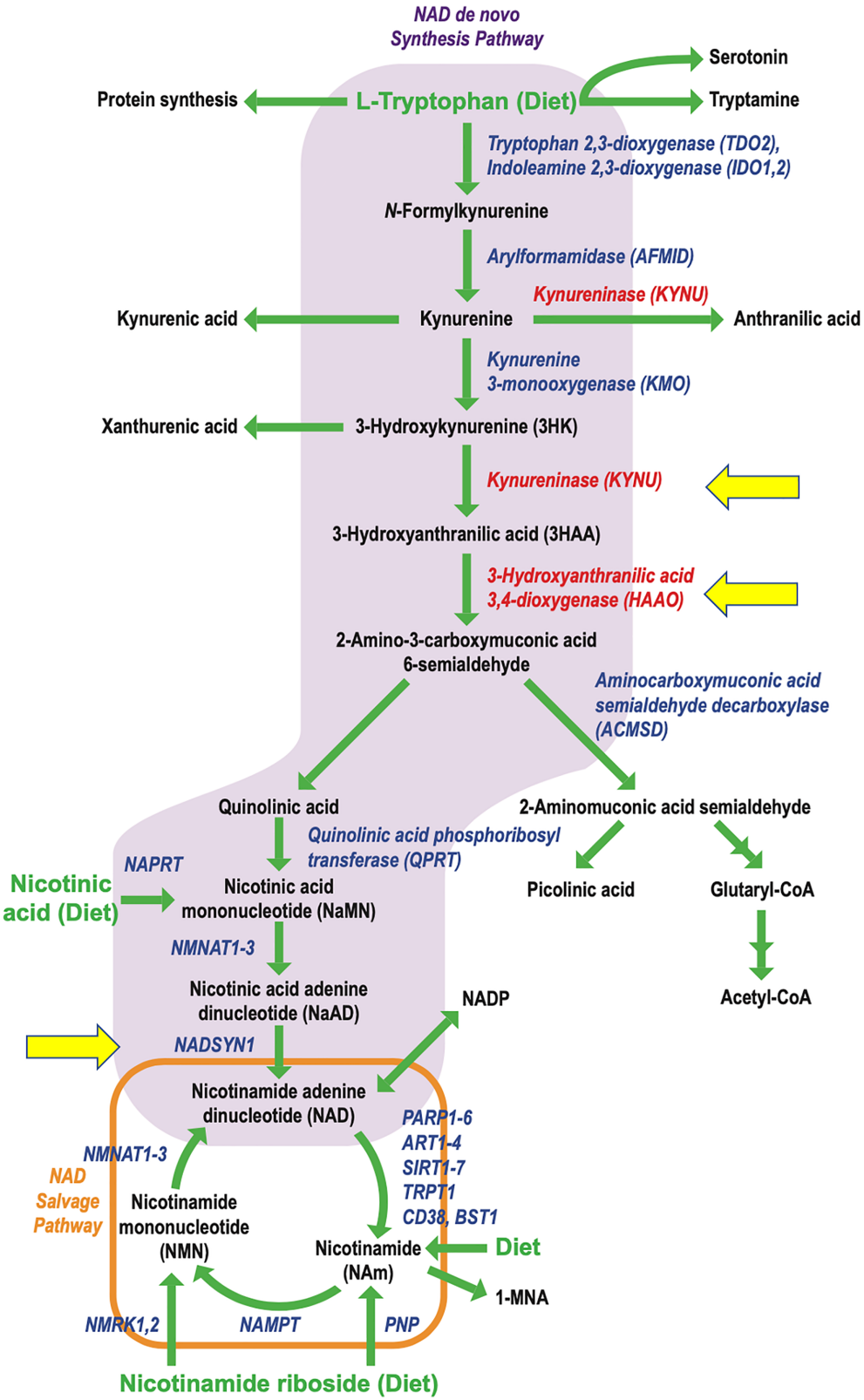

FIGURE 2 The NAD+ de novo synthesis pathway and NAD+ salvage pathway. Yellow arrows depict known genes with biallelic pathogenic variants in humans. Adapted from Shi, H., Enriquez, A., Rapadas, M., Martin, E. M. M. A., Wang, R., Moreau, J., Lim, C. K., Szot, J. O., Ip, E., Hughes, J. N., Sugimoto, K., Humphreys, D. T., McInerney-Leo, A. M., Leo, P. J., Maghzal, G. J., Halliday, J., Smith, J., Colley, A., Mark, P. R., Collins, F., Sillence, D. O., Winlaw, D. S., Ho, J. W. K., Guillemin, G. J., Brown, M. A., Kikuchi, K., Thomas, P. Q., Stocker, R., Giannoulatou, E., Chapman. G., Duncan, E. L., Sparrow, D. B., Dunwoodie, S. L. (2017). NAD deficiency, congenital malformations, and niacin supplementation. *New England Journal of Medicine*, *377* (6), supplementary appendix, p.16. Copyright © 2017 Massachusetts Medical Society. Reprinted with permission

syndrome, because of the phenotypic frequency of these four malformations (OMIM 617660, 617661). Although vertebral, cardiac, renal and limb defects tend to be the most common anomalies noted in these patients, additional findings include tracheoesophageal fistula, cleft palate, intrauterine growth restriction with short stature, microcephaly, and global developmental delays (Table 3). It is important to underscore there have been malformations described involving multiple human organ systems besides skeletal, limb, vertebral, and cardiovascular, including CNS, respiratory, gastrointestinal, endocrine, and sensory. For this reason, it has been suggested patients with

TABLE 2 Specific anomalies identified in mice with dietary restriction of tryptophan and niacin

Heart
Bicuspid aortic valve
Membranous ventricular septal defect
Muscular ventricular septal defect
Overriding aorta
Patent truncus arteriosus
Double outlet right ventricle
Vertebral and rib anomalies
Cervical vertebrae
Thoracic vertebrae
Lumbar vertebrae
Sacral vertebrae
Underdeveloped ribs
Kidneys
Hypoplasia or agenesis
Dysmorphic (duplex, hydronephrosis)
Limbs
Talipes
Digits
Polydactyly
Oligodactyly
Syndactyly
Skull
Exencephaly
Eyes
Coloboma
Microphthalmia
Cleft palate
Abdominal wall
Omphalocele
Gastroschisis
Caudal agenesis

Note: Adapted from Cuny et al. (2020), table S1, pp. 15–16.

genetic variants in the NAD+ de novo synthesis pathway have Congenital NAD Deficiency Disorder (CNDD), as NAD+ deficiency can impact development of all organs and systems, not just vertebrae, heart, kidneys, and limbs (Szot et al., 2020).

In addition, nine of the 15 families in which pedigrees were taken noted previous miscarriages, with four of the remaining six mothers having just one pregnancy at time of evaluation. Thus, nine of 11 multigravid mothers reported a miscarriage (Shi et al., 2017; Szot et al., 2020; Szot et al., 2021).

Evaluation of NAD levels in humans is difficult and complex. Currently, there is no clinical testing available to evaluate intracellular or plasma NAD levels. However, three of the patients in the original study had low plasma NAD levels confirmed on a research basis (Shi et al., 2017). Yeast models demonstrating the specific variants in patients with other *HAAO*, *KYNU*, and *NADSYN1* mutations all showed reduced NAD production (Szot et al., 2020; Szot et al., 2021). Thus, although there were genetic changes in genes creating enzymes in the NAD+ de novo synthesis pathway, the result was low NAD production, which is hypothesized to have caused the congenital malformations in the affected patients.

2.3 | Review of malformations seen in mice and humans with NAD+ deficiency compared to multiple malformation conditions

The overlap of malformations seen in mice and the features found in multiple malformation conditions discussed here is striking. Examples include exencephaly, abdominal wall defects and limb defects seen in LBWC, cardiac and abdominal wall defects seen in POC, omphalocele and spine defects noted in OEIS, and vertebral, cardiac, renal, and limb anomalies required in the criteria for VACTERL and seen in all these conditions. Whether the caudal agenesis noted in the mice truly overlaps with the genitourinary, anal, and lower limb findings of OEIS, MURCS, and sirenomelia has not been fully evaluated in mouse embryos to date but could potentially be similar.

As NAD+ is synthesized through tryptophan catabolism in the liver, the maternal contribution of NAD+ in early embryonic development is crucial until the embryo/fetus can take over this process. In the mouse models, wild type maternal NAD levels were distinctly reduced during pregnancy (Cuny et al., 2020). In humans, reduced levels of NAD+ precursors in the blood during all trimesters of pregnancy seem to be common, even with nondeficient diets and ample dietary vitamin intake (Baker et al., 2002; Sha et al., 2022). These findings indicate that the risk of developing NAD+ deficiency is elevated during pregnancy, the time when sufficient maternal NAD+ supply is required to ensure normal embryonic development.

Although humans with biallelic variants in *HAAO*, *KYNU*, and *NADSYN1* have multiple malformations which originated in the embryonic period of development, I hypothesize there are findings which develop during the fetal period also. These include poor somatic growth and GDD/ID attributable to abnormal brain development after the embryonic period.

Patients with biallelic variants in HAAO, KYNU, and NADSYN1 are unable to synthesize a sufficient amount of NAD+ themselves during the fetal period. Other children with multiple malformation conditions hypothesized to be caused by lack of maternal NAD+ contribution in early pregnancy are able to make their own NAD+ once their liver has developed adequately. Thus, patients who have developmental anomalies more dependent primarily on the maternal contribution of NAD+ during the embryonic period may not have these other findings which occur during the fetal period of growth and development.

In addition to affecting early embryonic development, all the multiple malformation conditions mentioned here have been identified in discordant twins (Achiron et al., 2000; Adam et al., 2020; Xu et al., 2018). Because of this, the RCEM Study Group suggests the shared pathogenesis

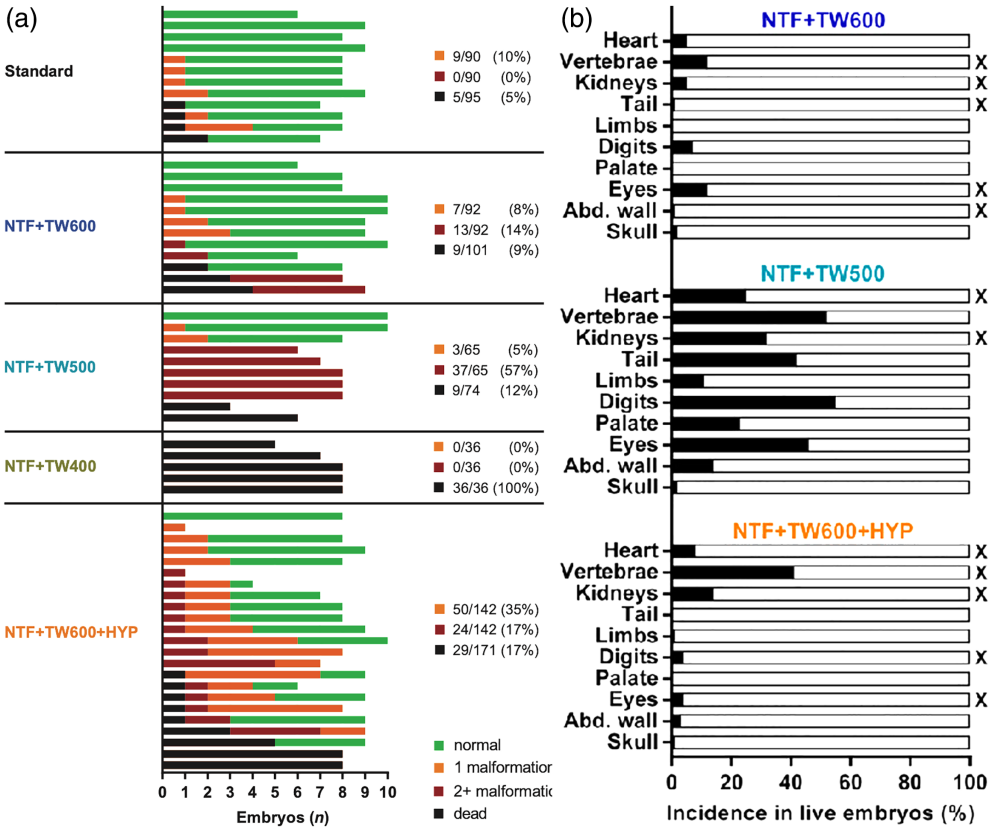

FIGURE 3 (a) Phenotypes of C57BL/6J wild-type mouse embryos at E18.5, within the maternal diet treatment groups, as indicated on the left. Each horizontal bar represents a litter and length of the bars indicates the total number of embryos per litter. All dead embryos were found to be early resorptions. Total counts and percentages of embryos within each treatment group are summarized on the right. NTF, vitamin depleted and tryptophan-free feed; TW, tryptophan-supplemented water, 400–600 mg/L. HYP-hypoxia at E9.5 (8% O_2, 8 h). From Cuny, H., Rapadas, M., Gereis, J., Martin, E. M. M. A., Kirk, R. B., Shi, H., Dunwoodie, S. L. (2020). NAD deficiency due to environmental factors or gene–environment interactions causes congenital malformations and miscarriage in mice. *Proceedings of the National Academy of Sciences, United States of America*, *117*(7), 2020, supplementary appendix, p.6, figure S3. Reprinted with permission. (b) Types of NAD deficiency malformations found in mice with dietary restrictions ± hypoxia. From Cuny, H., Rapadas, M., Gereis, J., Martin, E. M. M. A., Kirk, R. B., Shi, H., Dunwoodie, S. L. (2020). NAD deficiency due to environmental factors or gene–environment interactions causes congenital malformations and miscarriage in mice. *Proceedings of the National Academy of Sciences, United States of America*, *117*(7), p. 3741, figure 1. Reprinted with permission

causing the spectrum of multiple malformation conditions discussed here must explain the following (see Sections 3.4–3.6; Adam et al., 2020).

2.4 | An increased rate of reported discordant monozygotic twinning and instances of co-occurrence of two phenotypes in one individual or in a co-twin

The mice studied with NAD deficiency demonstrated intra-litter variability (some unaffected, some with one malformation, some with two or more malformations, some with embryonic losses). Although mice are polyovulatory, which would not explain if the splitting process could affect discordant embryonic development in human monozygotic twins, the offspring in these studies are genetically identical due to inbreeding, suggesting this could be a proper model of discordant monozygotic twinning. A plausible mechanism for these findings could be either inadequate maternal production of NAD+ to support a multiple gestation, or asymmetric NAD+ distribution to the developing embryos through the placenta. This could also explain reports of multiple malformations in dizygotic twins and triplets (including IVF pregnancies; Adam et al., 2020; Wijers et al., 2013). There was also individual mouse variability, where genetically identical mice with the same environmental exposure show unique combinations of phenotypic features. Additionally, NAD+ could be important in the mechanism which causes conjoined twins.

2.5 | There is minimal to no recurrence risk of these specific conditions

In humans, a study of 87 patients with VATER/VACTERL showed no increased in the overall prevalence of component features in first degree relatives (Bartels et al., 2012). In the mouse models with decreased NAD levels, there is extreme variability both intra-litter (same gestation) and inter-litter, where the same conditions for genetically identical mice produce litters in which some mice are affected

TABLE 3 Anomalies seen in affected patients with known biallelic variants in *HAAO*, *KYNU*, and *NADSYN1*

Skeletal—vertebral, rib, polydactyly, missing digits, hyperphalangism, micromelia
Cardiac—hypoplastic left heart, tetralogy of Fallot, Shone syndrome, others (ventricular septal defect, atrial septal defect, patent ductus arteriosus)
Urinary—renal agenesis (bilateral and unilateral), renal dysplasia, ureter agenesis
Neurologic (brain)—cerebellar, microcephaly, hydrocephalus, seizures, global developmental delay, intellectual disability, autism
Neuromuscular—spinal dysraphism/tethered cord, talipes, arthrogryposis, pterygia, muscular hypotonia
Respiratory—hypoplastic lung, laryngeal web
Gastrointestinal—TEF, polysplenia, pyloric stenosis, anterior anus
Endocrine—hypothyroidism, hypoparathyroidism
Sensory—sensorineural hearing loss, inner ear abnormalities, ocular crystals, hypopigmented iris with nodules
Integumentary—syndactyly
Lymphatic—nuchal redundancy, thickening, cystic hygroma
Facial dysmorphisms
Cleft soft palate, microretrognathia
Other—bilateral single palmar crease, joint hypermobility
Short stature

Note: Adapted from Shi et al. (2017), Szot et al. (2020), Ehmke et al. (2020), Schüle et al. (2021), Szot et al. (2021), and L. Bird, personal communication, September 14, (2021).

and entire litters in which no mice are affected. This could potentially be explained by larger maternal mice buffering circulating NAD+ precursor levels more effectively, and inconsistent rates of food and water ingestion (Cuny et al., 2020). In humans, there could certainly be improved or altered dietary intake or absorption of the NAD+ precursors tryptophan and niacin in subsequent pregnancies. In addition, pathophysiologic factors such as inflammation, type 2 diabetes, aging, and obesity could influence NAD+ availability (Cuny et al., 2020; Zhang & Ying, 2019).

2.6 | A lack of a known recurrent genetic cause in affected individuals

As has been explained above, wild type maternal mice with environmental influences of diet and hypoxia produced offspring with all the features of multiple malformation conditions included in this article. Thus, a genetic cause in affected humans would not be necessary to produce the malformations seen with maternal NAD+ deficiency. Interestingly, maternal *Haao* LoF variants exacerbated effect of dietary restriction on embryonic development in mice (Cuny et al., 2020). How human maternal heterozygous variants in NAD+ de novo synthesis pathway genes influence NAD+ production and thus impacts human embryonic development has yet to be conclusively determined.

In summary, the discordant anomalies in twins, along with the different multiple malformation conditions discussed above, should not be considered as discrete conditions, but viewed as a single entity, similar to how aspen tree stems appear to be individual but are all part of a single organism, connected by one underground root system. I hypothesize the connection for all these conditions is NAD+ deficiency (Figure 4). The term Congenital NAD Deficiency Disorder (CNDD) could be considered for patients with these malformations; however, it is important to emphasize there have been no confirmatory experimental studies in humans to confirm this hypothesis.

3 | BODY WALL, AMNION, AND UMBILICAL CORD ABNORMALITIES

When discussing NAD+ deficiency as a mechanism for specific malformations, the timing of the NAD+ deficiency can have a major impact on the specific anomalies generated by this deficiency. Perhaps the most evident example of this is the impact on umbilical cord and body wall development. There are three important recurring anomalies to review: body wall closure defects, amniotic band development and consequences, and umbilical artery development and anomalies.

Briefly, the amniotic cavity develops above the bilaminar embryonic disc (Carnegie stage 5, 7–8 days of development; Vermeij-Keers et al., 1996), and is lined by amniocytes. The amniotic cavity enlarges and encloses the embryo. In the ninth week of development, the amniotic membrane attaches itself to the chorion and the extraembryonic cavity disappears (Hartwig et al., 1991).

The embryo continues to grow in relation to the connecting stalk. The cranial and caudal pole of the embryo revolve under its ventral surface, and the umbilical ring, the thoracic wall, and the abdominal wall obtain their ventral position (Vermeij-Keers et al., 1996). Parts of the allantois and yolk sac are incorporated within the embryo, taking the positions of the primitive urogenital sinus/urinary bladder and the rectum respectively (Figure 5a; Hartwig et al., 1991).

Perhaps the most visible difference between the commonly labeled multiple malformation conditions discussed here is the presence or absence of a body wall defect. In normal development, the lateral body folds, formed from lateral plate mesoderm with overlying ectoderm, begin to grow ventrally (Sadler & Feldkamp, 2008). Cranial and caudal folds also form, and the combination of all four body folds narrows the connection between the yolk sac and embryonic endoderm (Sadler & Feldkamp, 2008). Depending on the timing of the body wall closure defect, the size of the defect in relation to the embryo along with the internal organs involved will vary (Figure 5b). Thus, regarding body wall formation LBWC, POC, and OEIS should be considered a spectrum with the abdominal wall and evisceration findings dependent on the timing of the NAD+ deficiency.

The amniotic membrane can be damaged at any time during embryogenesis. Gastroschisis is proposed to occur secondary to amniotic rupture along the umbilical cord in its pars flaccida between 6 and

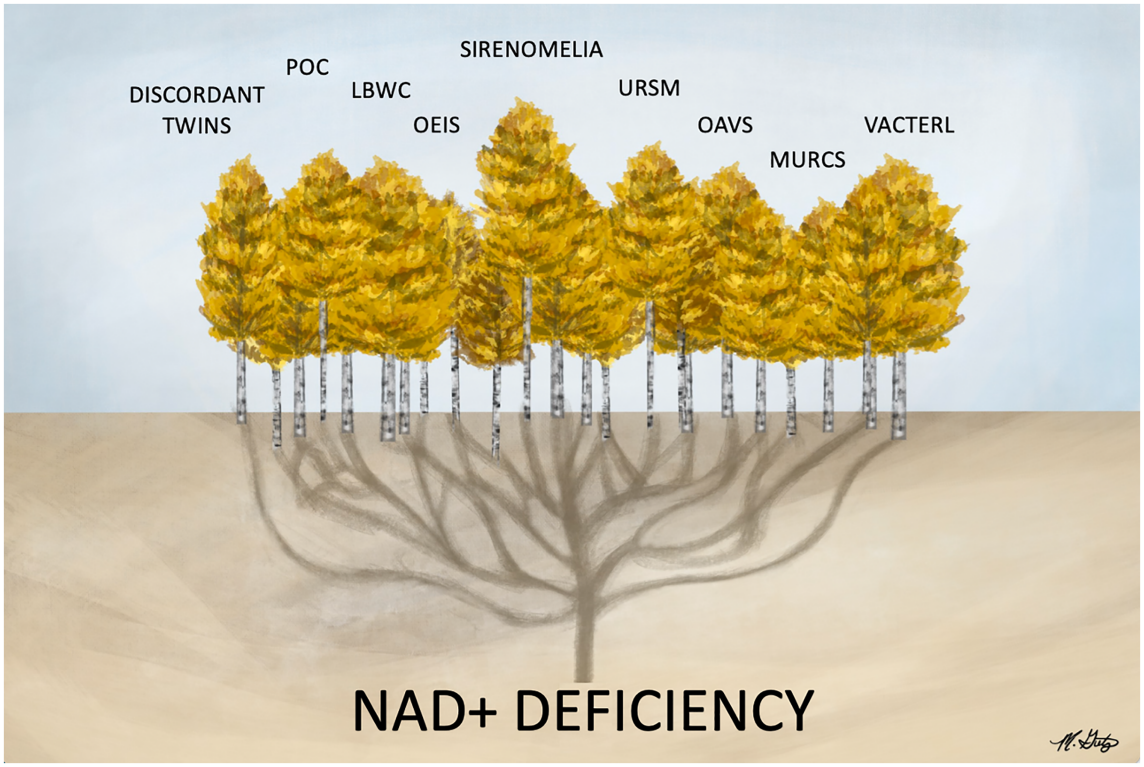

FIGURE 4 Visual analogy of recurrent multiple malformation conditions discussed here, including discordant twin anomalies, demonstrating my hypothesis these are not discrete conditions, but are part of a single entity, all unified by the mechanism of NAD+ deficiency. LBWC, limb-body wall complex; MURCS, Mullerian duct aplasia-renal anomalies-cervicothoracic somite dysplasia; OAVS, oculoauriculovertebral spectrum; OEIS, omphalocele-exstrophy-imperforate anus-spinal defects; POC, pentalogy of Cantrell; URSM, urorectal septum malformation; VACTERL, vertebral-anal-cardiac-tracheoesophageal fistula-renal-limb

9 weeks after conception (Bargy & Beaudoin, 2014). Although damage to the amnion resulting in amniotic bands is commonly associated with LBWC, there are cases of LBWC without amniotic bands (Craven et al., 1997) and there are cases with amniotic bands with or without exencephaly with no body wall defects (Halder, 2010). In addition, there are reports of POC with amniotic bands (Schüppler et al., 1994; Peer et al., 1993). Also, there are multiple reports of amniotic bands occurring with limb or craniofacial defects with body wall or exencephaly/encephalocele (López-Muñoz & Becerra-Solano, 2018). Thus, amnion damage can occur in addition to other anomalies or in isolation, and the timing and location of the NAD+ deficiency will determine the final phenotypic outcome.

Regarding umbilical cord development, the endoderm lining the primary yolk sac is surrounded by mesenchyme, a portion of which differentiates into the vitelline arteries and veins (Blackburn & Cooley, 2006). The umbilical cord is thus formed by the fusion of yolk sac derivatives and connecting stalk mesenchyme. Normally, the first organ to form from the primary yolk sac in humans is the allantois. In conditions in which the allantois does not develop or is lost early in embryogenesis, the allantoic arteries do not develop with the connecting stalk. The insult most likely occurs prior to 23 days gestation (9 days postconception; Blackburn & Cooley, 2006). This can result in persistent vitelline artery, believed to be the primary etiology of sirenomelia (Stevenson, 2021). Single umbilical artery (SUA) is posited to be caused either due to primary agenesis or atrophy of an existing vessel (Blackburn & Cooley, 2006). SUA has been seen in all the multiple malformation conditions discussed here, along with being an isolated anomaly (Arteaga-Vázquez et al., 2019; Duncan & Shapiro, 1993; Hunter et al., 2011; Jnah et al., 2015; Suri et al., 2000; Wheeler & Weaver, 2001).

4 | DISCUSSION

Until now, the paradigm for human multiple malformations has been viewed primarily through the lens of genetic impact. Indeed, pleiotropy is defined as the phenomenon of a single gene locus influencing two or more distinct phenotypic traits (Stearns, 2010). The assumption has been that if the DNA is abnormal (chromosomal anomalies, CNVs, SNVs) the subsequent production of RNA and proteins will be abnormal also. However, regarding malformations, there has been little evaluation of the processes of replication, transcription, translation, and post-translational modification themselves when DNA is normal. NAD+ is a vital, unrecognized partner to DNA in building cells. A helpful analogy is as follows: In building cells or organisms, if DNA is considered the blueprint, NAD+ is important in providing labor and materials necessary for the replication and development of cells and organisms. The energy (labor) is created through the formation ATP,

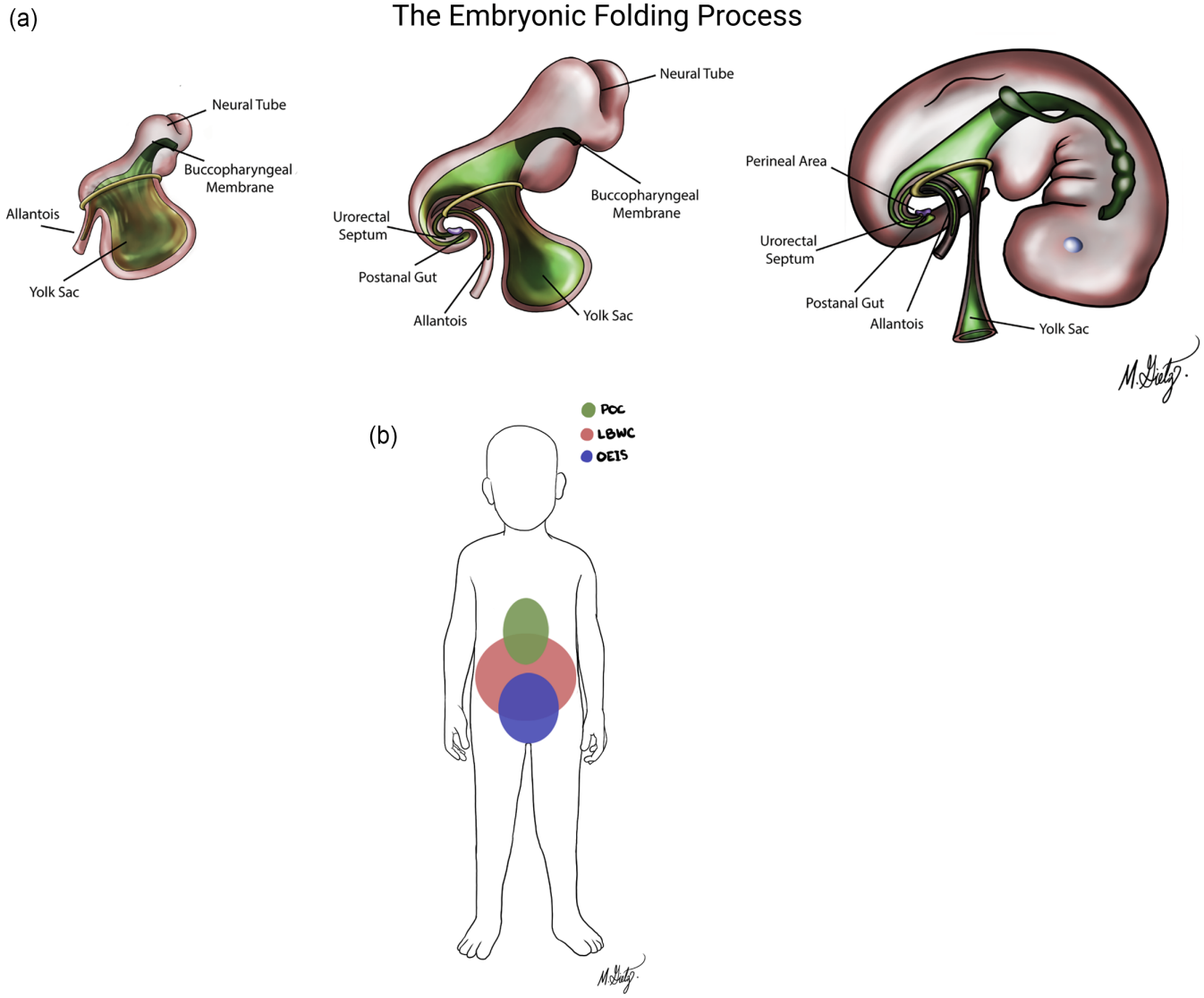

FIGURE 5 (a) The embryonic folding process demonstrating how the embryo grows in relation to the body stalk. The diameter of the umbilical ring (gold in the image) is the same in all three embryos of Carnegie stages 9, 11, and 13. In this way, the umbilical ring decreases in size relative to the embryo. Used with permission of Elsevier Science & Technology Journals, adapted from Hartwig, N. G., Steffelaar, J. W., Van de Kaa, C., Schueler, J. A., Vermeij-Keers, C. (1991). Abdominal wall defect associated with persistent cloaca. The embryologic clues in autopsy. *American Journal of Clinical Pathology*, *96*(5), p. 645, figure 5. Permission conveyed through Copyright Clearance Center, Inc. (b) Demonstration on how timing of NAD+ deficiency could impact body wall formation due to closure of body wall in a cranial to caudal fashion

which requires NAD+ for its synthesis. NAD+ is a co-factor and/or substrate (material) for over 300 enzymes (Sahar et al., 2011), which include sirtuins and PARPs, that are vital for the processes of replication, transcription, translation, and post-translational modification. NAD+ is also critical for normal mitochondrial function and many metabolic processes (Cantó et al., 2015). Thus, looking at human development and disease through the lens of NAD+ deficiency opens unique insights into the mechanisms of disease and abnormal development.

There are many human malformations, which occur both individually and in combination, for which a mechanism, genetic or otherwise, has yet to be identified (Innes & Lynch, 2021). Historically, there have been hypotheses presented to identify potential mechanisms for these conditions. In the original article on VATER association, Quan and Smith suggested a common type of defect in mesoderm could be the basis for nonrandom association (Quan & Smith, 1973). Opitz et al. (2002) suggested defects in blastogenesis as the mechanism for these malformations. Lubinsky (2017) proposed embryonic hypocellularity as an explanation these malformations, which he also noted were associated with monozygotic twinning, maternal diabetes, some forms of aneuploidy, and certain mitochondrial disorders. Most recently, Stevenson (2021) proposed vitelline vascular steal as the common pathogenesis for sirenomelia, OEIS complex, limb-body wall defect, and other malformations of caudal structures. All these mechanisms could be caused by NAD+ deficiency because NAD+ acts at the (intra)cellular level, and

TABLE 4 List of anomalies and conditions for which NAD+ deficiency is a potential mechanism

Acardia
Amelia
Amniotic bands
Arthrogryposis
Autism
Bladder exstrophy
Bowel atresias/tracheoesophageal fistulas
Brain anomalies
Chromosomal abnormalities
Cleft lip and palate
Cloacal exstrophy
Clubfoot
Conjoined twins
Cryptorchidism
Cystic hygroma
Diaphragmatic hernia
Discordant twins
Endocrine abnormalities
Gastroschisis
Heart defects
Hip dysplasias
Holoprosencephaly
Hydrops
Hypospadias
Intellectual disability/developmental delay
LBWC
Limb anomalies
Lung anomalies
Microcephaly
Microphthalmia
Microtia
Moebius syndrome
MURCS
Neural tube defects
OAVS
OEIS
Omphalocele
POC
Poland anomaly
Polydactyly/syndactyly
Pseudotrisomy 13
Renal agenesis/Potter sequence
Rib anomalies
Sensory defects
Sirenomelia
Steinfeld syndrome
URSM
Thomas syndrome
Vertebral anomalies

therefore affects all tissue types, all organ systems, and cellular growth and division. As it is variable in its quantity and areas of influence, the timing and location of its deficiency are critical in determining what malformations are ultimately the result of its impact. For these reasons, I hypothesize that NAD+ deficiency, either through decreased availability or synthesis, represents a potential mechanism for these malformations (Table 4).

Although the primary discussion thus far has been regarding decreased NAD+ production through the NAD+ de novo synthesis pathway, there are adult conditions which could result in decreased maternal availability of NAD+ during pregnancy. Aging, obesity, and diabetes decrease the amount of NAD+ available for necessary cellular processes (Zhang & Ying, 2019). Diabetic embryopathy overlaps multiple malformation conditions discussed here in the following anomalies: congenital heart defects, vertebral, renal, and limb defects, anal anomalies, caudal dysgenesis, hemifacial microsomia, cleft lip ± cleft palate, neural tube defects, sirenomelia, and urorectal septum malformation (Castori, 2013). Pregestational diabetes is listed as a risk factor for VACTERL anomalies, along with assisted reproductive technologies (ART), and chronic obstructive pulmonary disease, all of which could cause anomalies through diminished NAD+ (Adam et al., 2020; Lubinsky, 2017; van de Putte, van Rooij, Haanappel, et al., 2020). Thus, NAD+ deficiency is a plausible mechanism for diabetic embryopathy.

Neural tube defects have been reported with genetic causes along with environmental causes including teratogens, obesity, and diabetes, and have been associated with multiple malformation conditions and other anomalies including holoprosencephaly, diaphragmatic hernia, cardiac, renal, limb, genitourinary and gastrointestinal defects, among others (Dean et al., 2020). Folic acid supplementation has reduced the incidence of neural tube defects by over 70% (MRC Vitamin Study Research Group, 1991). However, the importance of NAD/NADP in folate metabolism has not been extensively studied as a potential mechanism for neural tube defects which continue to occur with or without folic acid supplementation (Zheng & Cantley, 2019).

Holoprosencephaly can occur in isolation and in combination with neural tube defects and multiple other anomalies including craniofacial, limb, vertebral, cardiovascular, renal, and gastrointestinal (Siebert et al., 2005). Although approximately 66% of patients with holoprosencephaly have a chromosomal or single gene cause (Solomon et al., 2018), it can occur in conditions such as pseudotrisomy 13 and Steinfeld syndrome for which a molecular cause has not been identified (Kruszka & Muenke, 2018). Noting again a connection between environmental causes, including teratogens and maternal diabetes, and holoprosencephaly, NAD+ deficiency could be a plausible mechanism for holoprosencephaly when a genetic cause is not discovered.

NAD+ deficiency could impact aneuploidies through two potentially critical mechanisms. First, reduced NAD+ has been reported in aging and is a plausible mechanism for the increased rate of meiotic nondisjunction seen in older women of child-bearing age (Bertoldo et al., 2020; Wu et al., 2019). Second, a hypothesis could be made regarding the increased need for NAD+ as a substrate to perform replication, transcription, and translation of the additional genes found on the third copy of chromosomes 21, 18, and 13 in viable trisomies (Jackson et al., 2018), leading to decreased cellular availability of NAD+. Thus, the increasing severity of anomalies and poor growth of these infants could be due to decreased NAD+ availability to perform the usual cellular and organism functions dependent on NAD+.

In addition to causing aneuploidy, NAD+ deficiency through its impact on sirtuins and PARPS can affect DNA repair mechanisms, including base excision repair and nucleotide excision repair, nonhomologous end joining, and homologous recombination (Mei et al., 2016). Also, NAD+ has an important role in DNA methylation, including through SIRT1 (Zhang & Kraus, 2010). Thus, in addition to causing nongenetic disease and anomalies, the role of NAD+ deficiency in genetic disease itself should be considered.

5 | FUTURE DIRECTION

When looking at human development through the lens of NAD+ deficiency there are many plausible areas of potential research. The main categories involve the maternal and fetal contribution of NAD+ during development. Regarding the maternal contribution, studying NAD levels in women both pregnant and nonpregnant, women with recurrent miscarriage, pregnancies with multiple gestation along with singletons, and mothers with additional conditions such as diabetes, obesity, and advancing age would be valuable. Regarding supplementation with NAD+ precursors to improve NAD+ levels in pregnant mothers, there have been many studies on mice showing improvements in chronic disease parameters, but human studies are still limited, with no data on intracellular NAD+ levels (Hong et al., 2020). Additionally, targeting hyperactivated NAD+ consumption and NAD+ salvage and recycling may need to be considered to improve human NAD+ levels (Conlon & Ford, 2022). Studying NAD levels in children and patients with trisomies and potentially even deletions such as 4p- and critical single nucleotide variants with known poor growth and developmental delay could be valuable. Lejeune considered Down syndrome to be metabolic (Caracausi et al., 2018), and autism has been studied for metabolic disturbances including NAD+ alterations (Maes et al., 2019), thus the impact of NAD+ on these conditions could be evaluated in more depth. It may be interesting to investigate the impact of NAD+ deficiency on phenomena such as reduced penetrance and variable expressivity. Due to the increased rate of VACTERL anomalies and twin discordancy with ART, studying NAD levels in patients undergoing ART could prove valuable. Also, attempting to determine why vertebral, cardiac, renal, and limb malformations are the most common defects seen with NAD+ deficiency would be beneficial in learning how this molecule impacts development. Additionally, studying the impact of teratogens at the level of cellular NAD+ would be an interesting area of research. The overall contribution of NAD+ perturbations to the environmental component of gene/environment interaction could provide important insights into this phenomenon. Finally, a plan for comprehensive, unified research on the impact of NAD+ on human development and disease should be considered.

ACKNOWLEDGMENTS

I would like to gratefully acknowledge Sally Dunwoodie for her thoughtful review of this manuscript, her research group for their pioneering discovery of NAD+ deficiency and malformations, and for the gracious use of Figure 3a,b in this manuscript. I would also like to acknowledge John Carey for his insights and assistance in improving this manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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How to cite this article: Mark, P. R. (2022). NAD+ deficiency in human congenital malformations and miscarriage: A new model of pleiotropy. *American Journal of Medical Genetics Part A*, *188*A:2834–2849. https://doi.org/10.1002/ajmg.a.62764