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ORIGINAL ARTICLE

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Maleic acid is a biomarker for maleylacetoacetate isomerase deficiency; implications for newborn screening of tyrosinemia type 1

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Abstract

Dried blood spot succinylacetone (SA) is often used as a biomarker for newborn screening (NBS) for tyrosinemia type 1 (TT1). However, false-positive SA results are often observed. Elevated SA may also be due to maleylacetoacetate isomerase deficiency (MAAI-D), which appears to be clinically insignificant. This study investigated whether urine organic acid (uOA) and quantitative urine maleic acid (Q-uMA) analyses can distinguish between TT1 and MAAI-D. We reevaluated/measured uOA (GC–MS) and/or Q-uMA (LC–MS/MS) in available urine samples of nine referred newborns (2 TT1, 7 false-positive), eight genetically confirmed MAAI-D children, and 66 controls. Maleic acid was elevated in uOA of 5/7 false-positive newborns and in the

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three available samples of confirmed MAAI-D children, but not in TT1 patients. Q-uMA ranged from not detectable to 1.16 mmol/mol creatinine in controls ($n = 66$) and from 0.95 to 192.06 mmol/mol creatinine in false-positive newborns and MAAI-D children ($n = 10$). MAAI-D was genetically confirmed in 4/7 false-positive newborns, all with elevated Q-uMA, and rejected in the two newborns with normal Q-uMA. No sample was available for genetic analysis of the last false-positive infant with elevated Q-uMA. Our study shows that MAAI-D is a recognizable cause of false-positive TT1 NBS results. Elevated urine maleic acid excretion seems highly effective in discriminating MAAI-D from TT1.

KEYWORDS

maleic acid, maleylacetoacetate isomerase deficiency, tyrosinemia type 1, newborn screening, succinylacetone

1 | INTRODUCTION

Tyrosinemia type 1 (TT1, OMIM; #276700) is a rare inborn error of amino acid metabolism, caused by a deficiency of the enzyme fumarylacetoacetate hydrolase (FAH). Lack of FAH causes a subsequent intracellular buildup of the toxic metabolites fumarylacetoacetate, succinylacetoacetate, and succinylacetone (SA).[1] Left untreated, FAH deficiency causes severe clinical problems including renal tubular dysfunction, episodes of porphyria crises with neuropathy, and liver pathology such as liver failure, hepatocellular carcinoma, or hepatoblastoma, which have a high mortality.[1,2]

Because of its severity and the benefits of early diagnosis and treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC),[1,3] TT1 was added to the Dutch newborn screening (NBS) program in 2007, using dried blood spot (DBS) tyrosine (Tyr) as screening marker. This, however, resulted in many false-positive outcomes and subsequent termination of the program 3 months after its initial introduction.

In 2008, TT1 was reintroduced to the national NBS program using DBS SA as a marker.[4,5] While SA was considered to be pathognomonic for TT1,[5–7] false-positive screening results are still observed.[8–11] Since 2008, 62 newborns from the Dutch NBS have been referred for follow-up due to a positive SA screening result. Only 27 were found to have TT1, yielding a false-positive rate of 56.5%. Among the newborns referred to our center,[9] the false-positive rate is even higher (7/9 or 78%).

A 2017 study by Yang et al.[12] showed that in six children, elevated SA concentrations were due to maleylacetoacetate isomerase deficiency (MAAI-D, OMIM; #617596). MAAI, another enzyme in the Tyr catabolic pathway, catalyzes the conversion of maleylacetoacetate to fumarylacetoacetate. As a result, in MAAI-D, there is an accumulation of maleylacetoacetate and maleylacetone, but not of fumarylacetoacetate. It is likely that maleylacetoacetate is reduced to SA in the same way as its *cis*-isomer fumarylacetoacetate in TT1, thus causing (mildly) elevated SA in MAAI-D children.

We hypothesized that in children with false-positive TT1 screening results, elevated SA could be attributed to MAAI-D. Contrary to TT1, MAAI-D is unlikely to cause clinical problems.[12] Therefore, finding these children is an unwanted side-effect of TT1 screening, conceivably causing stress in families of these newborns and possibly resulting in unnecessary treatment of MAAI-D children with NTBC.

So far, genetic testing has been the only way to distinguish between TT1 and MAAI-D. In this study, we (re) evaluated urine organic acids (uOA) of referred newborns and genetically confirmed MAAI-D children. Thereafter, we further investigated a potential biomarker of MAAI-D, namely maleic acid (MA), by developing a liquid chromatography-mass spectrometry (LC–MS/MS) method for measuring quantitative urinary maleic acid (Q-uMA) concentrations. Q-uMA was then measured in available samples of the referred newborns, the confirmed MAAI-D children, and controls to confirm its value in the diagnosis of MAAI-D.

2 | METHODS

2.1 | Participant samples

Since 2008, nine newborns have been referred to the University Medical Center Groningen (UMCG) for a follow-up of a positive NBS SA result for TT1. Two of them (22%) were diagnosed with TT1, while seven (78%) had a

false-positive result. uOA chromatograms of the nine referred newborns, dating from the original referrals after the positive NBS result, were retrospectively investigated for the presence of MA. In still available urine samples, we measured Q-uMA.

Urine samples were also obtained for Q-uMA measurements from eight genetically confirmed MAAI-D children, who were originally referred after a positive SA screening result, and were later diagnosed with MAAI-D in different places (Quebec, Canada ($n = 5$); Naples, Italy ($n = 1$), Utrecht, The Netherlands ($n = 1$), and Montana, USA ($n = 1$)). When sufficient urine was available, we also performed uOA measurements in our laboratory.

Moreover, from the Québec NTBC Study Group, we received samples from two patients with mild hypersuccinylacetonemia due to so-called pseudo-deficiency alleles in the FAH gene, to investigate Q-uMA excretion. The five MAAI-D children and the two children with pseudo-deficient FAH alleles from Quebec have been described previously.[12,13]

In addition, we investigated Q-uMA excretion in 66 controls. Control samples consisted of anonymized urine samples from children investigated for suspicion of an inherited metabolic disease but with normal metabolic screening results.

From all referred newborns, we collected NBS and confirmatory Tyr and SA concentrations. From the genetically confirmed MAAI-D children, we requested available Tyr and SA concentrations, and *GSTZ1* gene variants from the collaborating laboratories.

The study design was in accordance with the current revision of the Helsinki Declaration. The need for formal ethical review was waived by the local ethics committee. Samples were collected and measured in accordance with the Dutch "Code of Good Use." Parents of all TT1 and (suspect) MAAI-D newborns gave informed consent.

2.2 | Laboratory analyses

uOA were retrospectively reevaluated in stored chromatograms, or analyzed in available samples (1000 μL) using gas chromatography-mass spectrometry (GC–MS). Urinary excretion of uMA was quantified in available urine samples (10 μL) using LC–MS/MS.

2.2.1 | Urinary organic acid analyses

A total of 1000 μL urine sample, internal standard solution (100 μL 4-phenylbutyric acid (0.5 mg/mL in 0.1 N HCl)) and 100 μL ethoxyamine HCl solution (200 mg/mL in H_2O) were pipetted in glass tubes and left to derivatize for 30 min at 60°C. A spatula tip of NaCl and two drops of HCl 37% were added, followed by extraction using ethyl-acetate/diethyl-ether (1/1 v/v). The organic layer, containing the ethoximized organic acids, was extracted and evaporated under a vacuum. A total of 200 μL derivatization reagent (BSTFA/pyridine/trichloro-methyl-silane 5/1/0.06 v/v/v) was added and left for derivatization for 30 min at 60°C. A total of 1 μL prepared samples were injected into the GC–MS. uOA were analyzed using a Thermo Scientific Trace 1310 gas chromatograph coupled to an ISQ LT single quadrupole mass spectrometer (Interscience, Breda, The Netherlands) operated in EI positive ionization mode using 70 eV and registered a total ion scan with an m/z range of 50–650. A (14% cyanopropylphenyl)methylpolysiloxane column (30 m × 0.250 mm × 0.25 μm film thickness, Restek Chromatography, Bellefonte, PA, USA) was used with helium as carrier gas (0.8 mL/min). Data acquisition was performed with Chromeleon Version 7.2 SR4 (Interscience, Breda, The Netherlands).

2.2.2 | Quantitative urinary maleic acid (Q-uMA) analyses

Q-uMA excretion was quantified using a new in-house LC–MS/MS method. MA, fumaric acid (FA), $^{13}C_2$-MA (internal standard), and formic acid were obtained from Sigma-Aldrich (Missouri, USA), and methanol was obtained from Biosolve (Valkenswaard, The Netherlands). FA was not quantified, but solely used to verify chromatographic separation from MA, as MA and FA are *cis*- and *trans*-isomers of 2-butenedioic acid, respectively. Urine was diluted to a creatinine concentration of 1 mmol/L. A total of 10 μL urine was mixed with 170 μL internal standard solution (0.59 μM $^{13}C_2$-MA in MilliQ), vortexed for 1 min, and centrifuged for 4 min at 20800 rcf. A total of 1 μL of supernatants were measured using an ultra-performance liquid chromatography (LC-30, Shimadzu, Japan) coupled to a triple quadrupole mass spectrometer (API 4500, Sciex, Ontario, Canada) with an electrospray ionization source. Analyses were carried out with an ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 μM) (Waters, Milford, MA, USA). The mobile phase consisted of 0.2% formic acid in Milli-Q (A) and 0.2% formic acid in methanol (B). The following gradient was applied: from 0% to 3.3% B in 2 min; from 3.3% to 100% B in 1 min; then 100% B is maintained during 1.5 min; then back from 100% to 0% B in 0.1 min and last 0% B is maintained for 1.8 min. The flow rate was 0.45 mL/min with a column temperature of 40°C. Detection was performed by negative ion electrospray ionization in multiple reaction monitoring modes. The following transitions in multiple reaction mode were used: m/z 114.8 → 71.0 for MA and FA, and m/z

116.8 → 73.0 for $^{13}C_2$-MA, with a dwell time of 350 ms. The declustering potential and collision energy were − 20 and − 15 V. The electrospray ionization source temperature was kept at 500°C and nitrogen was used as the nebulizing gas at 60 psi. The curtain gas and heater gas were set at 40 and 80 psi, respectively. The ion spray voltage was set at −4500 V. Data were analyzed using Analyst 1.6.3 and MultiQuant MD 3.0.3 (Sciex, Ontario, Canada).

Concentrations of MA were quantified using a calibration curve. Calibrators were prepared in Milli-Q water (Merck KaGA, Darmstadt, Germany) with a range of 0.5–200 μM. The method proved linear within this range with correlation coefficients of at least 0.999 over 6 calibration curves. Urine samples were spiked at two different concentrations. For each pooled sample, intra-assay variation was calculated from 10 replicates analyzed in a single analytical run. The intra-assay variation of urine MA was 2.3% (mean 1.7 μM) and 1.2% (mean 30.4 μM) respectively. Inter-assay variations were assessed by analyzing the two pool samples at 10 different days over a 3 months' period, showing variation coefficients of 3.0% (mean 1.7 μM) and 3.6% (mean 30.5 μM after exclusion of one outlier (6.0% including the outlier)). Recoveries varied between 101%–109% (1 μM) and between 99%–103% (30 μM). The limit of detection (LOD) was calculated by the formula LOD = (*3 * stdev of intercept*)/*mean of slope*. Limit of quantification (LOQ) was determined based on the concentration where the coefficient of variation was <20%. For this, samples with a MA concentration of 0.1, 0.2, 0.3, 0.4, and 0.5 μM were analyzed in triplicate in three batches, from which a CV was calculated. The LOD/LOQ was 0.12/0.20 μM.

2.2.3 | Genetic analyses

For all newborns with a false-positive NBS result who had available DBS samples, *GSTZ1* gene analysis was performed using next-generation sequencing. Briefly, DNA was isolated using the Protocol IQ Casework Pro Kit for Maxwell 16 (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The preparation and sequencing of the library were performed by GenomeScan BV (Leiden, The Netherlands). Samples were prepared according to the procedure for Hybridization Capture using the Agilent SureSelect XT HS Human All Exon V8 + NCV kit (5191–7409). The prepared libraries were sequenced using the Illumina NovaSeq6000 sequencer. Data processing and analysis were performed according to standard diagnostic procedures of the genome diagnostics section of the UMCG. Observed variants were verified using a variant description validation software (VariantValidator) and reported in compliance with the Human Genome Variation Society recommendations for describing sequence variants.[14]

3 | RESULTS

3.1 | Available samples

All uOA chromatograms of the nine referred newborns were available for reevaluation, and from three newborns, urine was available for Q-uMA measurements. All eight confirmed MAAI-D children had urine available for Q-uMA analysis, and for three, a sufficient urine volume was available to analyze uOA in our laboratory. Q-uMA was analyzed in all 66 controls. All urine samples were stored at −20°C prior to analysis, with no additional storage time for the sample from Montana, storage durations between 1 and 3 years for the Dutch and Italian samples, and storage between 4 and 6 years for the Quebec samples.

Figure 1 shows a simplified flowchart of results from available samples, while Table 1 summarizes all available results of NBS SA and Tyr concentrations, uOA and Q-uMA excretion, and the GSTZ1 gene analyses.

SA and Tyr concentrations collected from the NBS and at referral were significantly lower in MAAI-D than in TT1 (see Table 1). Other results are further described below.

3.2 | Urinary organic acids

Retrospective analyses of uOA chromatograms revealed the presence of elevated MA in five out of seven (5/7) referred newborns with false-positive TT1 NBS results (NBS-2, NBS-4, NBS-5, NBS-7, NBS-8). MA was not found in uOA chromatograms of the two TT1 patients (NBS-1 and NBS-2). In the three samples available for uOA analysis of the already confirmed MAAI-D children (MAAID-1, MAAID-2, and MAAID-3), elevated MA was demonstrated as well.

After the discovery of MA in MAAI-D, MA was added to the routine quantitative organic acid panel of our GC–MS method, using the calibration curve of the cis-isomer FA for quantification. MA in uOA is below LOD (<0.5 mmol/mol creatinine) in all samples measured since MA inclusion (>1000 samples), except in urines of individuals with known or suspected MAAI-D. The mass spectra of MA and maleylacetone under the given conditions and derivatization agents can be found in Figure S1.

3.3 | Quantitative urine maleic acid (Q-uMA)

Q-uMA excretion of 66 controls (mean age 2.30 years (range: 0.0–17.1); 58% males) ranged from not detectable to 1.16 (mean 0.41) mmol/mol creatinine. Q-uMA was not significantly related to gender ($p = 0.85$, using Mann Whitney U test) or age (Spearman $r = 0.042$, $p = 0.74$), yet there

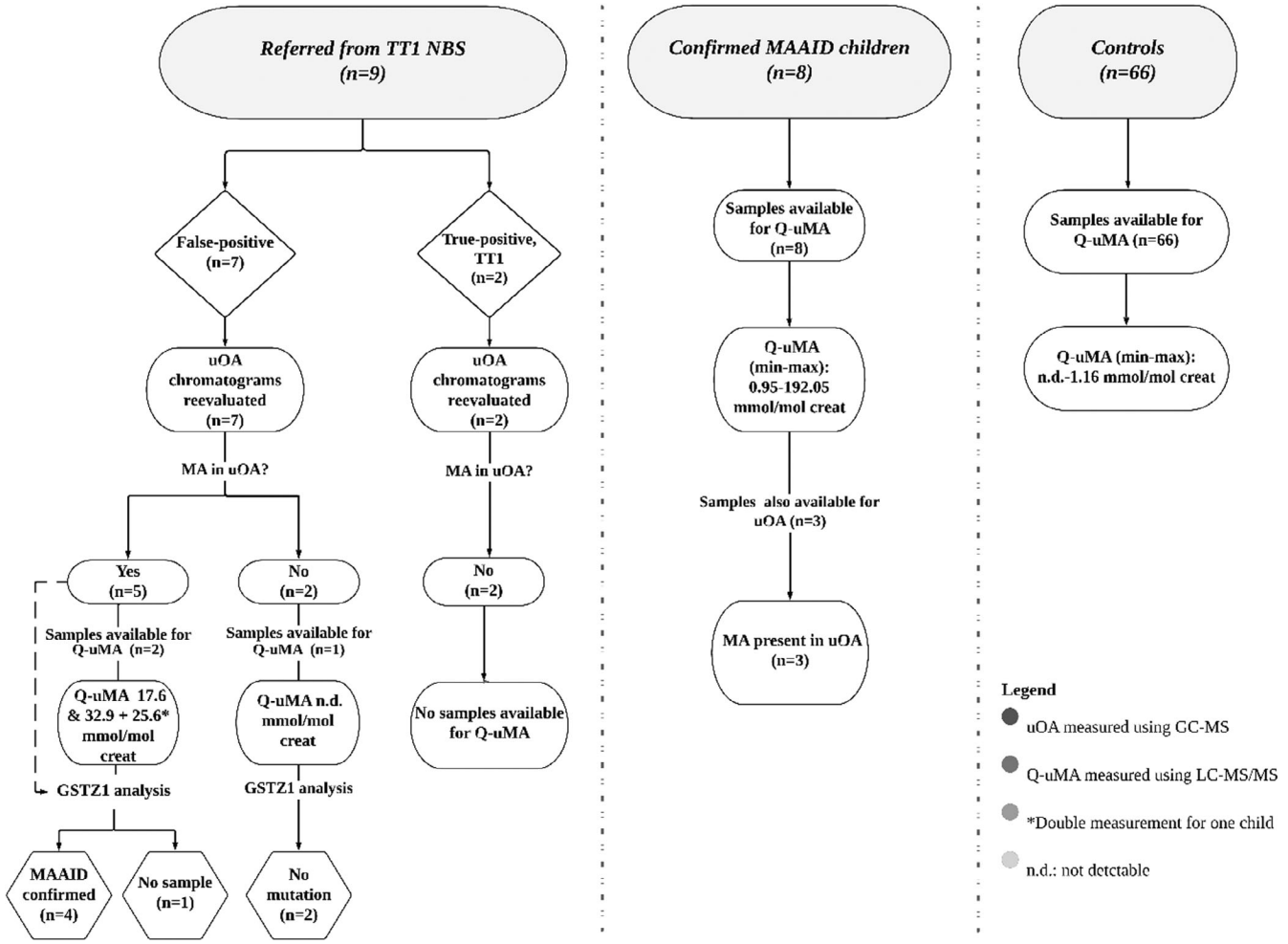

FIGURE 1 Simplified overview of available samples, analyses, and results.

seemed to be an increase of Q-uMA excretion up to 1 year of age, with a subsequent decrease thereafter (Figure S2).

Urine samples for Q-uMA measurements were available from three of the nine (3/9) referred newborns from the UMCG, and all eight MAAI-D children from other centers. Figure 2 shows the Q-uMA excretion of 12 samples from these 11 individuals (including two different samples from one child). Q-uMA excretion from the two false-positive children with elevated MA in uOA, and from the eight confirmed MAAI-D children ranged from 0.95 to 192.06 mmol/mol creatinine (Table 1). Q-uMA excretion in the sample of one child with absent MA in uOA was not detectable.

3.4 | FAH pseudo-deficient children

Lastly, we measured Q-uMA excretion in the samples of the two children with pseudo-deficient FAH alleles and requested SA and Tyr concentrations. Measured Q-uMA excretion was well within the control range, being <0.20 and 0.29 mmol/mol creatinine respectively. Urine SA excretions were 0.03 and 0.14 mmol/mol creatinine, plasma SA concentrations were 0.038 and 0.081 μmol/L, and plasma Tyr concentrations were 53 and 72 μmol/L, respectively.

3.5 | Genetic analyses

GSTZ1 analyses were performed in six out of seven referred newborns with a false-positive TT1 NBS result, as unfortunately, no DNA sample was available for the seventh. The variants identified in these newborns, as well as those in the previously confirmed MAAI-D children (including five previously described by Yang et al.[12]), are summarized in Table 1. Four out of the six newborns (4/6), all with elevated urine MA, were homozygous for the c.295G > A variant in the *GSTZ1* gene. This variant was also already found in two previously confirmed MAAI-D children (MAAID-2 and MAAID-8, although heterozygous in the latter case). In the two

TABLE 1 Summary of available newborn screening and confirmation results, uOA and uMA and genetic analyses of children who were referred to the UMCG with a positive newborn screening result, and results from children with confirmed MAAI-D. For the samples NBS-1-9, different analytical methods were used for SA quantification, namely: Neobase with Quattro Micro or Premier (NBS-1-5) and Neobase2 with Xevo TQD (NBS-6-9).

Sample	NBS results			NBS result	Confirmation results			Urine organic acids (GC/MS)			Urine MA (LC–MS/MS)	GSTZ1 analysis	Diagnosis
	DBS TYR µmol/L	DBS SA µmol/L	Cut-off value µmol/L		Plasma Tyr µmol/L	Urinary SA* mmol/mol creat	SA (DBS/plasma)* µmol/L	SA	MA	MAC	mmol/mol creat		
NBS-1	439	4.37	1.2	**TP**	388	51.20	NA	++	−	−	No sample	FAH: Ho c.554-1G > T	TT1
NBS-2	99	1.41	1.2	**FP**	63	0.63	NA	−	+	±	No sample	No sample available	MAAI-D**
NBS-3	493	5.13	1.2	**TP**	391	313.99	NA	++	−	−	No sample	FAH: He c.554-1G > T and c.674 T > G	TT1
NBS-4	266	1.96	1.2	**FP**	131	NA	DBS: 5.5	±	+	±	No sample	Ho. C.295G > A p.(Val99Met)	MAAI-D
NBS-5	86	2.03	1.2	**FP**	81	NA	DBS: 6.2	±	+	±	No sample	Ho. C.295G > A p.(Val99Met)	MAAI-D
NBS-6	96	1.13	0.9	**FP**	91	NA	DBS: <0.6	−	−	−	No sample	No mutation	None
NBS-7	85	0.83	0.6	**FP**	70	NA	DBS: 3.7 / 4.3	−	+	−	25.6/32.9	Ho. C.295G > A p.(Val99Met)	MAAI-D
NBS-8	10	0.66	0.6	**FP**	72	NA	DBS: 6.5	−	+	−	17.6	Ho. C.295G > A p.(Val99Met)	MAAI-D
NBS-9	68	1.26	0.6	**FP**	199	NA	DBS: 0.23	−	−	−	n.d.	No mutation	None
MAAID-1	36	2.08	<1.8	**FP**	62	NA	DBS: 1.18	+	++	++	107.30	He c.68-12G > A He c.464_471delTAGGAGAC insCTGGG	MAAI-D
MAAID-2	56	1.07	<0.6	**FP**	81	2.40	Plasma: 2.23	−	+	+	24.16	Ho. C.295G > A p.(Val99Met)	MAAI-D
MAAID-3	50	13.84	<5.42	**FP**	58	8.80	DBS: 4.91	+	+	+	192.06	c.16-1G > T; c.215C > G (p.Ser72Ter)	MAAI-D
MAAID-4	NA***	NA	NA	**FP**	NA	0.48	Plasma: 0.91	ND			23.38	He c.259C > T (p.Arg87Ter) He c.68-12G > A	MAAI-D
MAAID-5	NA	NA	NA	**FP**	99	0.14	Plasma:0.04	ND			2.25	Ho c.449C > T (p.Ala150Val)	MAAI-D
MAAID-6	NA	NA	NA	**FP**	67	0.10	Plasma: 0.04	ND			1.39	Ho c.449C > T (p.Ala150Val)	MAAI-D
MAAID-7	NA	NA	NA	**FP**	70	0.42	NA	ND			0.95	Ho c.449C > T (p.Ala150Val)	MAAI-D
MAAID-8	61	1.03	<0.7	**FP**	60	0.17	Plasma: 0.02	ND			2.72	He c.295G > A (p.Val99Met)	MAAI-D

Abbreviations: DBS, dried blood spot; FP, false positive; FAH, fumarylacetoacetate hydrolase; MAAI-D, maleylacetoacetate isomerase deficiency; MA, maleic acid; MAC, maleylacetone; NBS, newborn screening; NA, not available; n.d., not detectable; SA, succinylacetone; Tyr, tyrosine; TP, true-positive; TT1, tyrosinemia type 1.

*Reference values: for urine SA; <0.14 *m*mol/mol creatinine (Utrecht), < 0.06 *mmol/mol creatinine* (Montana), and <0.034 mmol/mol creatinine (Quebec). For plasma SA <0.024 µmol/L (Quebec) and <0.16 µmol/L (Utrecht).**Not confirmed.

***For samples MAAI-D-4-7 NBS results are not available as, at that time, SA values were determined through indirect colorimetric assay, and cannot be compared to MS/MS values. Tyr NBS results are also not available for those cases.

[Corrections added on 08 November 2023, after first online publication: Table 1 legend has been updated in this version.]

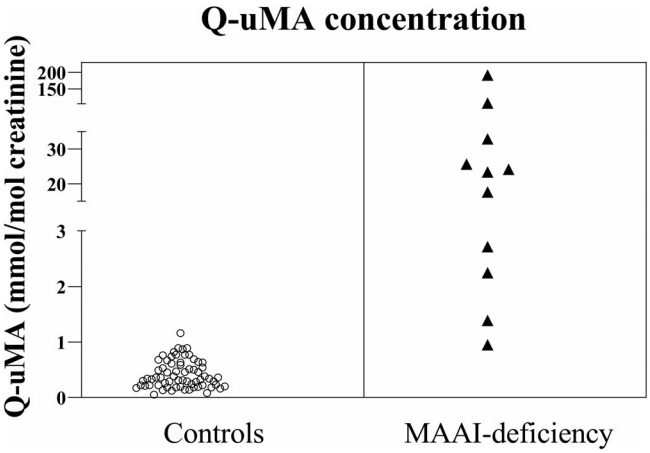

FIGURE 2 Urinary maleic acid concentrations in controls and MAAI-deficient children.

newborns with undetectable MA excretion (2/6 referred newborns; NBS-6 and NBS-9), no variants were identified in *GSTZ1*.

4 | DISCUSSION

In this study, we show that MAAI-D is a frequent, recognizable cause of false-positive TT1 NBS results based on elevated SA, for which Q-uMA appears to be an adequate biomarker.

In five out of seven (5/7) newborns with a false-positive NBS result, MA was clearly elevated (both in uOA and Q-uMA). From these newborns, four out of six (4/6) were genetically confirmed to have MAAI-D (no sample available for the seventh). In samples from previously confirmed MAAI-D children, Q-uMA was also clearly elevated, whereas, in TT1 patients, MA could not be identified. Therefore, Q-uMA is an interesting MAAI-D biomarker, that could serve as a secondary test to exclude MAAI-D following a positive SA NBS result.

MA is presumably primarily formed due to the accumulation of maleylacetoacetate (Figure 3). It can be hypothesized that the enzyme FAH, which converts fumarylacetoacetate into FA and acetoacetate, also catalyzes the formation of MA (the *cis*-isomer of FA) and acetoacetate from maleylacetoacetate. As FAH is deficient in TT1, MA will not be elevated in TT1 patients, which was confirmed in the TT1 patients in our study.

From the referred false-positive newborns that were genetically confirmed to have MAAI-D, all had the same homozygous variant (c.295G > A) in the *GSTZ1* gene. This variant was also found in the sample of MAAID-2 and was first seen in one child from the study of Yang et al.[12] (also included in this study, MAAID-8, see Table 1), who showed that this variant is associated with a low MAAI activity when expressed in bacteria. This missense variant (p.Val99Met) changes a conserved amino acid and is predicted to be deleterious by the PolyPhen2[15] and SIFT programs,[16] but not by Mutation Taster.[17] It is classified as a likely pathogenic variant by criteria of the American College of Medical Genetics.[18–20] This variant is more frequently reported in European populations compared to other populations.[20] Two children with undetectable MA excretion in uOA had no variants in *GSTZ1*, which further strengthens the association between the variant c.295G > A and the elevated MA levels.

Until now, MAAI-D has only been sparsely investigated and therefore its clinical relevance remains somewhat unclear. An abstract from 2016 discussed a single genetically confirmed case of MAAI-D.[21] This child presented with developmental delay, behavioral issues, irritability, and autistic features at 18 months of age, but the authors concluded that a causal relation between MAAI-D and the symptoms was not proven. As far as we know, this abstract is the first to report the possible association with MAAI-D and the presence of MA in urine. The largest cohort of MAAI-D children was described in 2017 by Yang et al.[12] and consisted of six children identified through NBS with mild hypersuccinylacetonemia. All children who had elevated SA concentrations, albeit lower than usually observed in TT1 patients, were genetically confirmed to have MAAI-D. Urine samples of five of these children were also included in this study, all showing elevated concentrations of MA in urine. None of these children have received any treatment (NTBC and/or diet), but nevertheless have all remained asymptomatic[12]; no kidney and liver dysfunction or disease was observed even up to, at least 13.3 years of age. Moreover, since 2017, none of the children still seen in the clinic have detectable liver-related changes on imaging or routine liver function tests (*Mitchell GA, personal communication*). This asymptomatic course of MAAI-D was already previously suggested by Fernandez-Cañon et al.[22] in 2002, who investigated MAAI-D in a mouse model. The MAAI-D mice did not appear to develop any clinical problems under normal circumstances. However, when fed a high protein diet, renal and liver damage occurred. Based on the study of Yang et al.,[12] it seems unlikely for humans in developed countries to experience similar protein overload (nutritional or catabolic) severe enough to cause adverse effects.[22] Although the evidence currently remains limited, these reports combined suggest that MAAI-D causes few to no clinical problems, substantiating the idea that treatment (or even monitoring) is unnecessary.

Of itself, MAAI-D does not belong in the NBS panel as a primary target, but given its shared informative biomarker

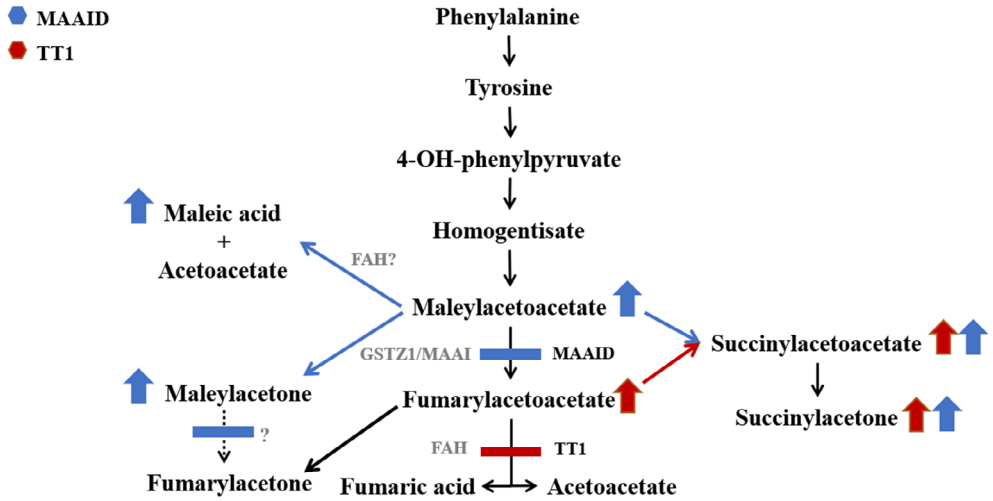

FIGURE 3 The catabolic pathway of tyrosine: This figure illustrates the effects of Tyrosinemia type 1 (TT1) by deficiency of fumarylacetoacetate hydrolase (FAH) and the effects of maleylacetoacetate isomerase (MAAI) deficiency (MAAID).

with TT1, it is a potentially identifiable condition on NBS. It may be important to rule out MAAI-D to prevent unnecessary treatment of children with NTBC, which does not only have adverse financial consequences, given that NTBC is an expensive drug, but should also be avoided because of the possible side effects that may arise from NTBC treatment. Follow-up testing of MA in urine, in conjunction with GSTZ1 analysis, may be used to confirm a false-positive TT1 screening result or, to rule out MAAI-D.

Higher SA and Tyr concentrations in our TT1 patients compared to MAAI-D underline the previous finding that both SA and Tyr are (usually) higher in FAH deficiency than in MAAI-D.[12] However, using SA and Tyr concentrations to lower the number of false-positive results in TT1 screening may not be advisable, as this might result in missing patients with milder forms of TT1 that could have only slightly elevated concentrations of SA.[23–25] This was illustrated by our results from the two children with pseudo-FAH deficiency who had both lower SA and Tyr concentrations than TT1 patients, and Q-uMA concentrations within normal range.

It should be noted that MA is not a specific marker for MAAI-D. MA concentrations are also elevated in patients treated with medication in the form of maleate salts, sodium dichloroacetate (DCA), and the DCA precursor dichloroaldehyde.[26] DCA and dichloroaldehyde inactivate MAAI,[22,27] mimicking MAAI-D, thereby elevating MA. It is suggested that this is more likely to occur in patients with a polymorphism in a drug-metabolizing enzyme, resulting in slow metabolism of MA.[26] Patients' drug history should be taken into account when interpreting urine MA excretion. However, the relevance of this may be limited for NBS, as newborns are unlikely to be treated with these medications. Hence, we believe that analysis of MA is of added value following elevated DBS SA concentrations in NBS.

For the moment, for measurements of (Q-)uMA, a child with a positive SA NBS for TT1 still needs to be referred for urine analysis. However, it is plausible that quantification of MA in DBS could allow MA to be used directly as a true second-tier test in DBS for TT1 NBS, thus avoiding unnecessary referrals of newborns with MAAI-D. Therefore, the next step is to further investigate MA quantification in DBS.

Lastly, this study has some limitations that should be addressed. First, our current sample size is relatively small, and our results should be replicated in a larger sample to substantiate our findings. Second, as this was a retrospective study, most urine samples had been stored for multiple years. The long-term stability of MA and the effect of freeze/thaw cycles on MA in urine is unclear. Storage of the samples could have possibly influenced the measured concentrations. This might be most relevant in the samples from Quebec (MAAID-4-8), which were stored the longest (4–6 years), and had also undergone more than one freeze–thaw cycle. Especially samples MAAID-5–8 only showed relatively small elevations of Q-uMA, resulting in a slight overlap of MA excretion in controls and MAAI-D. However, the genotypes of these children are also predicted to permit higher residual activity of MAAI than the genotypes of MAAID-4 and of the other newborns included in this study.[12] The children from Quebec were also older at the time of sample collection. We do not yet know whether the excretion of MA decreases with age in MAAI-D, as was seen with plasma and urinary SA during long-term follow-up without intervention.[12] However, as MA excretion seemed to decrease after 1 year of age in our controls (Figure S2), this may also hold true for children with MAAI-D.

Second, there might be some uncertainty in the range of Q-uMA concentrations for controls. The highest measured Q-uMA excretion among controls was 1.16 mmol/mol creatinine, yet this value seemed to be an outlier (see Figures 2 and Figure S2). There was no clear reason to exclude this child from the control

population, causing a slight overlap between MAAI-D cases and controls. The ranges of Q-uMA values in controls and MAAI-D cases, and the relevant cut-off value remain to be established using fresh samples collected and measured shortly after referral of an infant with a positive TT1 NBS result.

To conclude, we used urinary MA concentrations to identify MAAI-D and distinguish between MAAI-D and TT1. (Q-)uMA shows great potential for improving the NBS for TT1, yet further research into identifying and quantifying MA in DBS is necessary before a true second-tier test could be implemented in the national NBS program. Until then, observation of elevated MA by means of uOA or targeted Q-uMA analysis easily identifies MAAI-D in newborns with a false-positive TT1 test result based on elevated SA concentrations.

AUTHOR CONTRIBUTIONS

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FJvS, member of the Dutch Program committee for Newborn Screening and chair of the Dutch committee for Newborn Screening for Metabolic Diseases, has received investigator-initiated grants from SOBI. Other authors had nothing to disclose.

DATA AVAILABILITY STATEMENT

Data are available on reasonable request.

ETHICS STATEMENT

The study design was in accordance with the current revision of the Helsinki Declaration. The need for formal ethical review was waived by the Institutional Medical Ethics Committee of the University Medical Center Groningen (METc code 2021.289). Samples were collected and measured in accordance with the Dutch "Code of Good Use." Parents of all TT1 and (suspect) MAAI-D newborns gave informed consent.

PATIENT CONSENT STATEMENT

Parents of all TT1 and (suspect) MAAI-D newborns gave informed consent.

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SUPPORTING INFORMATION

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