Amniotic fluid gamma-glutamyl transferase for prediction of biliary atresia in cases of non-visualisation of the fetal gallbladder: a retrospective study using a validated analytical platform and local reference range

Tommy YT Cheung, Natalie KL Wong, Daljit Singh Sahota, Shreenidhi Ranganatha Subramaniam, SL Lau, X Zhu, WT Lui, Edwin KW Chan, Yvonne KY Kwok, KW Choy, TY Leung, Michael HM Chan, Felix CK Wong #, YH Ting #*

ABSTRACT

Introduction: The level of amniotic fluid gamma-glutamyl transferase (AFGGT) may help identify biliary atresia (BA) in cases of non-visualisation of the fetal gallbladder (NVFGB). This study aimed to validate a serum/plasma matrix–based gamma-glutamyl transferase (GGT) assay for amniotic fluid (AF) samples, establish a local gestational age–specific AFGGT reference range, and evaluate the efficacy of AFGGT for predicting fetal BA in pregnancies with NVFGB using the constructed reference range.

Methods: The analytical performance of a serum/plasma matrix–based GGT assay on AF samples was evaluated using a Cobas c502 analyser. Amniotic fluid gamma-glutamyl transferase levels in confirmed euploid singleton pregnancies (16^{+0} to 22^{+6} weeks of gestation) were determined using the same analyser to establish a local gestational age–specific reference range (the 2.5th to 97.5th percentiles). This local reference range was used to determine the positive predictive value (PPV) and negative predictive value (NPV) of AFGGT level <2.5th percentile for identifying fetal BA in euploid pregnancies with NVFGB.

Results: The serum/plasma matrix–based GGT assay was able to reliably and accurately determine GGT levels in AF samples. Using the constructed local gestational age–specific AFGGT reference range, the NPV and PPV of AFGGT level <2.5th percentile for predicting fetal BA in pregnancies with NVFGB were 100% and 25% (95% confidence interval=0, 53), respectively.

Conclusion: In pregnancies with NVFGB, AFGGT level ≥2.5th percentile likely excludes fetal BA. Although AFGGT level <2.5th percentile is not diagnostic of fetal BA, fetuses with AFGGT below this level should be referred for early postnatal investigation.

Hong Kong Med J 2024;30:218–26
https://doi.org/10.12809/hkmj2210567

[1,2] **TYT Cheung,** MB, ChB
[3] **NKL Wong,** MB, ChB, MRCOG
[3] **DS Sahota,** PhD
[1] **SR Subramaniam,** MB, ChB
[3] **SL Lau,** MB, ChB, MRCOG
[3] **X Zhu,** MB, BS, PhD
[3] **WT Lui,** MPhil
[4] **EKW Chan,** FHKAM (Surgery), FRCSEd (Paed)
[3] **YKY Kwok,** PhD
[3] **KW Choy,** PhD
[3] **TY Leung,** MD, FRCOG
[5] **MHM Chan,** FHKCPath, FHKAM (Pathology)
[5,6] **FCK Wong #,** MB, BS, FRCPA
[3] **YH Ting #*,** FHKAM (Obstetrics and Gynaecology), FRCOG

[1] *Department of Chemical Pathology, The Chinese University of Hong Kong, Hong Kong SAR, China*
[2] *Department of Chemical Pathology, Princess Margaret Hospital, Hong Kong SAR, China*
[3] *Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Hong Kong SAR, China*
[4] *Division of Paediatric Surgery and Paediatric Urology, Department of Surgery, Prince of Wales Hospital, Hong Kong SAR, China*
[5] *Department of Chemical Pathology, Prince of Wales Hospital, Hong Kong SAR, China*
[6] *Division of Chemical Pathology, Department of Pathology, Queen Mary Hospital, Hong Kong SAR, China*

# Equal contribution

* Corresponding author: tingyh@cuhk.edu.hk

This article was published on 5 Jun 2024 at www.hkmj.org.

New knowledge added by this study

- A serum/plasma matrix–based gamma-glutamyl transferase (GGT) assay reliably and accurately determines GGT levels in amniotic fluid samples.
- Using amniotic fluid gamma-glutamyl transferase (AFGGT) level <2.5th percentile to identify biliary atresia (BA) in cases of non-visualisation of the fetal gallbladder (NVFGB), the negative and positive predictive values were 100% and 25%, respectively.

Implications for clinical practice or policy

- A local gestational age–specific AFGGT reference range (the 2.5th to 97.5th percentiles) is available for clinical use.
- In pregnancies with NVFGB, AFGGT level ≥2.5th percentile likely excludes fetal BA; although AFGGT level <2.5th percentile is not diagnostic of BA, it is an important indicator of the need for early postnatal investigation.

羊水內γ-谷氨酰轉移酶濃度預測胎兒膽囊未顯示的膽道閉鎖：基於經驗證的化驗分析平台和本地參考範圍的回顧性研究

張爾庭、黃琨朗、邵浩達、SR Subramaniam、劉素玲、朱曉帆、呂慧婷、陳健維、郭家賢、蔡光偉、梁德楊、陳浩明、黃子鍵、丁婉霞

引言：羊水內γ-谷氨酰轉移酶（AFGGT）濃度可能有助於在胎兒膽囊未顯示的情況下識別膽道閉鎖。本研究的目的是在羊水樣本中驗證基於血清/血漿的γ-谷氨酰轉移酶（GGT）測定方法，建立本地胎齡特異的AFGGT參考範圍，並使用該參考範圍評估AFGGT預測胎兒膽囊未顯示妊娠中胎兒膽道閉鎖的有效性。

方法：本研究使用Cobas c502分析儀評估基於血清/血漿GGT測定法在羊水樣本中使用的表現。我們在確認整倍體單胎妊娠（孕16^{+0}週至孕22^{+6}週）中使用同一分析儀測定羊水樣本中GGT的濃度，用於確定本地胎齡特異的GGT參考範圍（第2.5至第97.5百分位數）。我們使用該參考範圍，確定在AFGGT濃度<第2.5百分位數時識別胎兒膽囊未顯示妊娠中胎兒膽道閉鎖的陽性預測值和陰性預測值。

結果：基於血清/血漿的GGT測定法能可靠且準確地檢測羊水樣本中GGT的濃度。使用構建的本地胎齡特異的AFGGT參考範圍，AFGGT濃度<第2.5百分位數預測在胎兒膽囊未顯示妊娠中胎兒膽道閉鎖的陽性和陰性預測值分別為100%和25%（95%置信區間=0, 53）。

結論：在胎兒膽囊未顯示妊娠中，AFGGT濃度≥第2.5百分位數可能可以排除胎兒膽道閉鎖；儘管AFGGT濃度<第2.5百分位數不能診斷胎兒膽道閉鎖，但低於該水平的胎兒應於產後早期進行相關檢查。

Introduction

Non-visualisation of the fetal gallbladder (NVFGB) in the second trimester is a rare condition affecting 0.1% of pregnancies.[1] It might be a transient finding—the gallbladder is visible later in pregnancy or after birth in 70% of cases.[2] Persistent NVFGB may be associated with benign conditions (eg, gallbladder agenesis); it may also be a manifestation of serious disorders, such as biliary atresia (BA), cystic fibrosis, or chromosomal abnormalities.[3] Although chromosomal abnormalities and cystic fibrosis can be identified prenatally via chromosomal microarray (CMA) and sequencing of the cystic fibrosis transmembrane conductance regulator gene, respectively, it remains challenging to diagnose BA before birth because no diagnostic prenatal test is currently available. Biliary atresia is a devastating condition and is the leading indication for liver transplantation in childhood.[4] Prenatal suspicion of BA allows prompt postnatal assessment and early diagnosis, permitting timely intervention via Kasai hepatoportoenterostomy and resulting in improved outcomes.[4]

The measurement of gamma-glutamyl transferase (GGT) in amniotic fluid (AF) has been suggested as a method for prenatal detection of BA. This transferase is secreted by the fetal biliary tract, passes into the intestines, and is ultimately excreted into the amniotic cavity. It becomes detectable in AF at around 14 weeks of gestation upon maturation of the intestinal villi and opening of the cloacal membrane.[5-7] Biliary tract obstructions, such as BA, hinder the passage of GGT into the intestines and AF, leading to reduced levels of amniotic fluid gamma-glutamyl transferase (AFGGT). Muller et al[5] first reported extremely low AFGGT levels in three fetuses with extrahepatic bile duct obstruction at 18 to 19 weeks of gestation; they concluded that a low AFGGT level could be a useful indicator of BA. Subsequently, Burc et al,[6] Dreux et al,[8] and Bardin et al[9] reported similar findings. Nevertheless, existing platforms for analysis of GGT in serum or plasma samples have not been thoroughly validated for use with AF samples. Furthermore, an appropriate reference range, essential for the interpretation of AFGGT results, is difficult to establish due to the limited availability of AF samples from normal pregnancies. Thus, publications regarding gestational age–specific reference ranges for AFGGT have been scarce.

Burc et al[6] established reference values for five AF enzymes (AFEs), including GGT, using the Hitachi 911 analyser (Roche Diagnostics). Despite a large sample size of 508, no separate reference range was constructed for each gestational age from 20 to 24 weeks, limiting clinical use of the findings.[6] Bardin et al[7] established another reference range for AFGGT, from 16 to 22 weeks, using the Integra 800 analyser (Roche). However, the numbers of samples at weeks 16, 21, and 22 were small and had relatively large standard deviations, precluding clinical application.[7] Both reference ranges were derived from Caucasian populations. Because the GGT level in adult blood varies according to ethnicity,[10] it is likely that AFGGT levels also vary according to ethnicity; thus, there is a need to establish a local AFGGT reference range. Furthermore, both previous reference ranges covered the 5th to 95th percentiles. A wider reference range, from the 2.5th to 97.5th percentiles, would allow greater flexibility in selecting cut-off values for clinical application.

In this study, we aimed to validate a serum/plasma matrix–based GGT assay for AF samples, establish a local gestational age–specific reference range for AFGGT (from the 2.5th to 97.5th percentiles), and evaluate the efficacy of AFGGT for predicting fetal BA in pregnancies with NVFGB using the constructed reference range.

Methods

This retrospective study used archived AF supernatant obtained from amniocentesis procedures that had been conducted to exclude fetal chromosomal abnormalities between January

2012 and December 2021. All amniocenteses were performed under ultrasound guidance with aseptic technique by fetal-maternal medicine specialists who also performed detailed ultrasound examinations to document the presence or absence of fetal structural abnormalities. All AF samples were centrifuged at 100 g for 10 minutes; 1 mL of the resulting supernatant was stored at -80°C in plain aliquots without additives (Axygen; BioGene, Union City [CA], United States), whereas the pellet was used for either CMA and/or karyotyping by G-banding analysis. The cytogenetic laboratory information system recorded the ultrasound findings, indication for amniocentesis, gestational age at amniocentesis, and CMA and/or karyotype results. Pregnancy and fetal outcomes, including the presence of any abnormalities at birth or autopsy findings, were recorded in each patient's electronic records.

Patients

Archived AF samples with known fetal and pregnancy outcomes, taken at 16^{+0} to 22^{+6} weeks of gestation from singleton pregnancies with a euploid fetus (confirmed by CMA or karyotype) during the period from January 2018 to December 2020, were retrieved to establish a gestational age–specific reference range for GGT. Pregnancies with fetal chromosomal, gastrointestinal, or hepatobiliary anomalies (particularly BA) polyhydramnios, or oligohydramnios were excluded to eliminate potential confounding effects of these conditions.

Non-visualisation of the fetal gallbladder was incidentally detected during fetal morphology scans in pregnancies with risk factors for fetal abnormalities because the fetal gallbladder was not assessed in low-risk routine anomaly scans, in accordance with the International Society of Ultrasound in Obstetrics and Gynecology guideline.[11] It was defined as failure to visualise the fetal gallbladder on two targeted ultrasound examinations performed 1 week apart. Isolated NVFGB was defined as NVFGB in the absence of other abnormal ultrasound findings. Pregnant women were counselled regarding possible differential diagnoses and offered amniocentesis for chromosomal analysis, as well as repeated ultrasound scans until the fetal gallbladder was visible. After birth, babies with persistent NVFGB were referred to paediatricians for hepatobiliary ultrasound and liver function tests. If the gallbladder was visible during prenatal scans, paediatricians did not order further tests in the absence of clinical suspicion. We followed the progress of all babies until the time of writing, using electronic hospital records for those delivered in public hospitals and phone calls for those delivered in private hospitals. With parental consent, post-mortem examinations were arranged in pregnancies terminated for serious associated fetal abnormalities.

Validation and analytical performance evaluation

Amniotic fluid samples were removed from -80°C storage in batches, thawed, and equilibrated to room temperature immediately prior to analysis. Gamma-glutamyl transferase levels were determined using an International Federation of Clinical Chemistry and Laboratory Medicine–standardised L-gamma-glutamyl-3-carboxy-4-nitroanilide (GGCNA) enzymatic colorimetric assay on a Cobas c502 analyser (Roche, Basel, Switzerland). Internal quality controls were performed before and after each batch.

Details of the AFGGT assay validation and analytical performance, including matrix effects; linearity; intra- and inter-run precision at various GGT levels; interference due to haemolysis, icterus, and lipaemia; and sample stability, are summarised in online supplementary Appendix 1.

Establishment of reference range

An AFGGT reference range was developed using the Generalised Additive Models for Location (μ), Scale (υ) and Shape (σ) [GAMLSS] package in R statistical software (version 3.3.2; R Foundation for Statistical Computing, Vienna, Austria). All GGT values were transformed to their natural log equivalent before model construction; the final model balanced between percentile smoothness, goodness-of-fit, and simplicity. Model fit was assessed using the generalised Akaike information criterion and by inspection of residuals with quantile-quantile plots for all measurements, detrending of quantile-quantile plots, and comparison of empirical percentiles to fitted percentiles. Empirical percentiles were determined for comparative purposes by grouping GGT levels according to gestational age (in weeks).

The final model was used to determine reference values for the 2.5th, 5th, 50th, 95th ($z = \pm 1.645$), and 97.5th percentiles ($z = \pm 1.964$). Percentiles were determined using the expression $\mu \times (1 + z_p \upsilon\sigma)1/\upsilon$, where z_p is the percentile of interest and μ, υ, and σ are dependent on the time covariate, gestational age. The GGT reference range was constructed using R statistical software and Microsoft Excel (Microsoft Corporation, Redmond [WA], United States).

We estimated a priori that 293 AFGGT measurements were needed to achieve a standard error of 10% of the gestational age–specific standard deviation for the 2.5th and 97.5th (z=1.96) reference percentiles, assuming that the standard error of the percentile of interest is expressed as a multiple of standard deviation using the formula $\sqrt[2]{(1 + 0.5z^2_{1-\alpha/2})/n}$, where each gestational age between 16^{+0} and 22^{+6} weeks has a minimum of 42 measurements.

Performance evaluation

Measured levels of AFGGT in pregnancies with

NVFGB were transformed to their gestational age–specific percentile values using the final model. We then determined the positive predictive value (PPV) and negative predictive value (NPV) of AFGGT level <2.5th percentile for identifying BA in euploid pregnancies with NVFGB.

Results

Validation and analytical performance

The performance of the GGT assay using AF is summarised in online supplementary Appendix 2. Validation studies indicated that the GGCNA enzymatic colorimetric assay for determination of GGT activity in AF had linearity, precision, recovery, interference profiles, and stability comparable to the values reported for measurement of GGT in plasma and serum samples. The verified analytical measurement range was 10 to 1200 U/L. No significant interference was observed in the presence of 0.25 g/dL haemoglobin, 103 μmol/L bilirubin, or 16.8 mmol/L triglyceride. The limits of haemolysis/icterus/lipaemia indices above which interference occurred were significantly higher than the degrees of those indices in all analysed samples.

Gestational age–specific reference range

A database search identified 518 stored amniotic samples (502 [97%] Chinese and 16 [3%] other Asian ethnicities) suitable for use in establishing a local gestational age–specific AFGGT reference range. The median number of samples per week was 65; there were 2 weeks with <42 samples (27 and 28 samples in the 16th and 19th week of gestation, respectively). The online supplementary Table lists the regressed values of AFGGT levels according to percentile for each week of gestation.

The simplest best-fit model indicated a linear relationship between natural log-transformed GGT and gestational age. Table 1 and the online supplementary Figure show the final smoothing equations for median, coefficient of variation, and skewness, along with gestational age–specific smoothed percentile curves and values of AFGGT for our local population. Residuals of the final model had a mean skewness of 0 and a variance of 1; they were almost mesokurtic, with kurtosis values close to 3.

Performance evaluation

The database search identified 32 pregnancies with NVFGB from 2012 to 2021, of which 18 had an available AF sample (four isolated NVFGB and 14 non-isolated NVFGB). There were no cases of cystic fibrosis. Nine cases were excluded from analysis, including five with chromosomal abnormalities, one with renal hypoplasia and oligohydramnios, one with hydrops and polyhydramnios, and two in which the biliary tract anatomy could not be identified. The nine remaining cases for analysis included four with isolated NVFGB and five with non-isolated NVFGB (Table 2). The median gestational age at amniocentesis was 21.0 weeks (interquartile range=20.7-22.1).

The Figure depicts the AFGGT levels in the nine pregnancies for analysis compared with our local gestational age–specific AFGGT reference range. Four cases had AFGGT level <2.5th percentile, including one with BA (AFGGT level of 27 U/L at 20^{+3} weeks) and three with transient non-visualisation (AFGGT levels of 32, 68, and 37 U/L at 20^{+0}, 20^{+6}, and 22^{+2} weeks, respectively). Five had AFGGT level ≥2.5th percentile, including one with gallbladder agenesis and four with transient non-visualisation (Table 2). Using AFGGT level <2.5th percentile as the cut-off, the NPV and PPV for identifying fetal BA in pregnancies with NVFGB were 100% and 25% (95% confidence interval=0, 53), respectively (Table 3). Repeated analysis using AFGGT level <5th

TABLE 1. Smoothing equations determined by modelling for median, coefficient of variation, and skewness used to compute z-scores and percentiles of the natural logarithm of gestational age–specific gamma-glutamyl transferase level

Model parameter	Equation
Median	$\mu(GA)=11.744090425 - 0.299528318 \times GA$
Coefficient of variation	$\sigma(GA)=e(-4.710556652 + 0.121033972 \times GA)$
Skewness	$\upsilon(GA)=15.99323025 - 0.70898993 \times GA$

Abbreviation: GA = gestational age

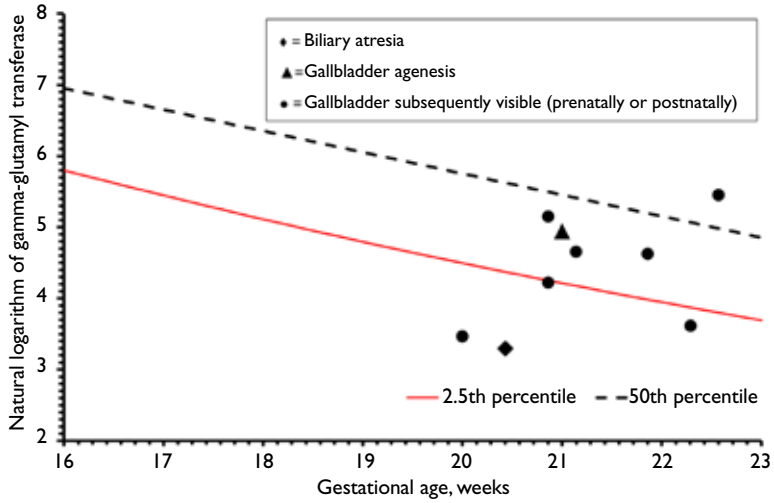

FIG. Amniotic fluid gamma-glutamyl transferase (AFGGT) levels in cases with non-visualisation of the fetal gallbladder compared with the gestational age–specific AFGGT reference range

TABLE 2. Characteristics of the nine cases of non-visualisation of the fetal gallbladder included in the current study*

Case No.	GA at diagnosis, wk	Additional USG findings	GA at amnio, wk	AFGGT, U/L	AFGGT GA-specific percentile	GB at follow-up	Postnatal investigations	Postnatal diagnosis	Age at progress tracking	Remarks
1	27	LCDH Porta hepatis cyst	20^{+3}	27	<2.5	Persistent NVFGB	USG GB CBD not visible, portal cyst 1.2 cm LFT elevated bilirubin HIDA scan biliary system patency not established	Biliary atresia	5 y	CDH repair Kasai operation Liver transplant at 2 y
2	22^{+2}	Hepatic hilar cyst	22^{+2}	37	<2.5	Persistent NVFGB	USG GB visible, choledochal cyst 1 cm LFT normal at 1 mo	Choledochal cyst	11 mo	Plan excision at 1 y
3	20^{+4}	Polydactyly Hypospadias	20^{+6}	68	<2.5	GB visible at 21^{+4} wk	USG small GB LFT normal at 8 mo	No hepatobiliary concern	13 mo	
4	20	Nil	20	32	<2.5	GB visible at 21 wk	Nil	No hepatobiliary concern	33 mo	
5	21	Nil	21	140	5-50	Persistent NVFGB	USG GBA LFT normal at 6 mo	No hepatobiliary concern	8 mo	
6	20^{+6}	Echogenic bowel	20^{+6}	172	5-50	Persistent NVFGB	LFT normal at 1 y	No hepatobiliary concern	5 y	
7	21	Nil	21^{+1}	105	5-50	GB visible at 23 wk	USG GB visible LFT normal at 1 mo	No hepatobiliary concern	34 mo	
8	21^{+1}	Nil	21^{+6}	102	5-50	GB visible at 32^{+3} wk	Nil	No hepatobiliary concern	13 mo	
9	21^{+2}	SUA Polydactyly	22^{+4}	233	50-95	GB visible at 22^{+2} wk	LFT normal at 3 y	No hepatobiliary concern	9 y	

Abbreviations: AFGGT = amniotic fluid gamma-glutamyl transferase; amnio = amniocentesis; CBD = common bile duct; CDH = congenital diaphragmatic hernia; GA = gestational age; GB = gallbladder; GBA = gallbladder agenesis; HIDA scan = hepatobiliary iminodiacetic acid scan; LCDH = left congenital diaphragmatic hernia; LFT = liver function test; NVFGB = non-visualisation of the fetal gallbladder; SUA = single umbilical artery; USG = ultrasonography

* Amniocentesis chromosomal microarray of all cases were normal and the outcome of all cases was live birth

percentile or the reference ranges from Burc et al[6] or Bardin et al[7] yielded identical results.

Discussion

Biliary atresia is a rare congenital anomaly, with a prevalence of 1 in 15 000 to 20 000 live births among Caucasian populations.[12] However, BA is more common in East Asians; the prevalence is 1 in 5000 to 7000 among Chinese populations.[13,14] Untreated BA is a progressive and devastating disease that can cause cirrhosis and death by 2 years of age.[14] This outcome can be prevented by early intervention via palliative Kasai hepatoportoenterostomy, which is essential for re-establishing biliary drainage. If biliary drainage cannot be re-established, liver transplantation is necessary. Indeed, BA is the most common indication for liver transplantation in children, contributing to 75% of transplantations in children before 2 years of age.[12] It is therefore imperative to diagnose BA early, preferably during the prenatal period. Nevertheless, prenatal diagnosis of BA is challenging because ultrasound cannot directly examine the patency of fetal bile ducts. When NVFGB is associated with a hepatic hilar cyst or heterotaxy, it is highly suggestive of BA.[15] Non-visualisation of the fetal gallbladder with a hepatic hilar cyst is an indicator of cystic BA, a rare subtype representing 5% to 10% of BA cases[16]; therefore, this prenatal combination is uncommon. Heterotaxy is another rare condition with a prevalence of 1 in 10 000 live births,[17] and concurrent BA is present in only 10.4% of left atrial isomerism cases[18]; therefore, this prenatal combination is even less common. Consequently, NVFGB may be the only prenatal sign indicating the possibility of BA. However, in cases of isolated NVFGB, it is difficult to differentiate between BA and gallbladder agenesis. The discovery of the association between low AFGGT levels and fetal BA has led to interest regarding the role of AFGGT in the management of NVFGB.

Validation and analytical performance

To our knowledge, this study is the first to validate the International Federation of Clinical Chemistry and Laboratory Medicine–standardised GGCNA enzymatic colorimetric assay on the Cobas c502

TABLE 3. Reported efficacies of amniotic fluid enzymes for the prediction of biliary atresia in cases of non-visualisation of the fetal gallbladder

	Biliary atresia	Normal	Sensitivity*	False positive rate*	Positive predictive value*	Negative predictive value*
Bardin et al[9]: 17-22 weeks of gestation†						
Abnormal amniotic fluid enzyme‡	3	1	100%	4%	75%	100%
Normal amniotic fluid enzyme	0	26				
Dreux et al[8]: <22 weeks of gestation†						
Abnormal amniotic fluid enzyme§	3	4	100%	20%	43%	100%
Normal amniotic fluid enzyme	0	16				
Dreux et al[8]: >22 weeks of gestation†						
Abnormal amniotic fluid enzyme§	1	5	20%	9%	17%	93%
Normal amniotic fluid enzyme	4	52				
Current study: 20-22^{+4} weeks of gestation						
Abnormal amniotic fluid enzyme‡ ¶	1	3	100%	37.5%	25%	100%
Normal amniotic fluid enzyme	0	5				
Current study: <22 weeks of gestation						
Abnormal amniotic fluid enzyme‡ ¶	1	2	100%	33.3%	33.3%	100%
Normal amniotic fluid enzyme	0	4				

* Calculation based on No. of cases reported in the corresponding article
† Abnormalities other than biliary atresia were excluded
‡ Gamma-glutamyl transferase level <5th percentile
§ Gamma-glutamyl transferase and/or intestinal alkaline phosphatase level <0.5 multiples of the median
¶ Gamma-glutamyl transferase level <2.5th percentile

analyser, a common locally available plasma and serum analyser, for use with AF. We have demonstrated that accurate and precise measurements of GGT can be achieved with AF samples, enabling adoption in clinical settings. We have also established that the analytical measurement range is 10 to 1200 U/L. This is particularly important for an AFGGT assay because AFGGT levels in euploid pregnancies can vary across multiple orders of magnitude, whereas plasma GGT levels in healthy individuals usually remain below 100 U/L. This verification of the lower limit of quantification and linearity range improves confidence in our measurements. Furthermore, we have excluded potential interference, particularly from haemoglobin, because AF samples may sometimes contain maternal blood. We were initially concerned about GGT stability because some samples had been stored for several years; however, consistent with the World Health Organization report that GGT is stable for years in frozen serum and plasma samples,[19] we found that AF stored frozen in plain bottles without additives at -20°C or -80°C remained stable for at least 6 months (online supplementary Appendix 1). This finding indicates that supernatants can be stored and subsequently retrieved for GGT assays, an important consideration if amniocentesis is performed in the early second trimester but an indication for AFGGT testing is identified during a mid-trimester morphology scan.

Gestational age–specific reference range

We have established a reference range for AFGGT levels at 16^{+0} to 22^{+6} weeks of gestation using a large local reference population of 518 samples (all Asian, 97% Chinese); this reference population is the largest compared with similar previous publications. The presence of an adequate sample size for each week of gestation allowed us to establish a reference range for each gestational age, thus overcoming the aforementioned limitations regarding clinical use of the two previous reference ranges.[6,7] With respect to the two previous reference ranges, the 5th, 50th, and 95th percentiles in our study are similar to those reported by Bardin et al[7] but higher at most gestational ages than those reported by Burc et al.[6] The larger sample size in our study permitted the calculation of the 2.5th and 97.5th percentiles, such that a 95% central reference range could be established; this allows greater flexibility in selecting cut-off values for clinical application.

It has been reported that AFGGT levels are increased in oesophageal atresia and duodenal atresia, whereas they are decreased in anal atresia without fistula.[20,21] Although duodenal atresia can easily be diagnosed prenatally by detection of the double bubble sign, this sign usually appears after 24 weeks of gestation.[22] Prenatal diagnosis of oesophageal atresia relies on the indirect sign of non-visualisation of the stomach bubble and

polyhydramnios; the sensitivities of these ultrasound findings range from 8.9% to 42%.[20] Additionally, prenatal detection of anal atresia without fistula depends on the absence of the perianal muscular complex, but the anal sphincter does not fully mature until after 28 weeks of gestation.[23] Further research regarding the use of AFGGT for early detection of these congenital gastrointestinal tract obstructions is valuable, and the availability of a local gestational age–specific reference range is crucial for supporting such research.

Performance evaluation

In the present study, the NPV and PPV of AFGGT level <2.5th percentile for identifying fetal BA in NVFGB were 100% and 25%, respectively (Table 3). Bardin et al[9] assessed the efficacy of low AFGGT levels in predicting BA among cases of NVFGB between 17 and 22 weeks of gestation. Of the 26 cases with AFGGT level ≥5th percentile, none had BA; of the four cases with AFGGT level <5th percentile, three had BA. The corresponding NPV and PPV were 100% and 75%, respectively. The PPV in their study may have been higher because amniocentesis was performed before 22 weeks of gestation in all cases.[9] In our cohort, after exclusion of the two cases in which amniocentesis was performed after 22 weeks of gestation, the PPV only marginally improved to 33.3%. Our figures are more consistent with those of Dreux et al,[8] who analysed the efficacy of AFEs for predicting BA in NVFGB before and after 22 weeks of gestation. In that study, abnormal AFE was defined as GGT and/or intestinal alkaline phosphatase level <0.5 multiples of the median.[8] Before 22 weeks of gestation, there were three cases of BA among seven cases with abnormal AFE and no cases of BA among 16 cases with normal AFE. The corresponding NPV and PPV were 100% and 43%, respectively. However, after 22 weeks of gestation, there was only one case of BA among six cases with abnormal AFE and four cases of BA among 56 cases with normal AFE. The corresponding NPV and PPV were 93% and 17%, respectively (Table 3). These findings confirmed the expected decrease in AFE efficacy for predicting BA after 22 weeks of gestation. By that gestational age, the passage of GGT from the intestine into the AF is impeded by mature anal sphincter muscles in normal fetuses; thus, the AFGGT level is very low after 22 weeks of gestation, and it is difficult to distinguish between a low level due to BA and a low level related to normal development.[5-7]

In our cohort, there were three fetuses without BA who had AFGGT level ≤2.5th percentile; one of these fetuses had an extremely low AFGGT level (Case 4) [Table 2]. In addition to its association with fetal BA, low AFGGT is linked to chromosomal abnormalities, cystic fibrosis, anal atresia without fistula, and polyhydramnios. However, none of the three fetuses had any of these conditions. Although one fetus (Case 2) [Table 2] had a small choledochal cyst which might have impeded biliary drainage and caused a mild decrease in AFGGT, we could not find a potential explanation for the low AFGGT levels in the other two fetuses.

Limitations

Limitations of our study include its retrospective nature and small cohort size. The incidence of NVFGB is low (0.1%).[1] In the largest systematic review concerning the outcomes of NVFGB, encompassing seven studies, the total number of cases was 280; among 170 cases of isolated NVFGB, only six (3.5%) had BA.[2] In Hong Kong, the fetal gallbladder is not routinely assessed in low-risk routine anomaly scans, in accordance with the International Society of Ultrasound in Obstetrics and Gynecology guideline.[11,24] Among the 32 cases of NVFGB in our cohort, one (3.1%) had BA; this incidence is comparable to the findings in the aforementioned review. However, only 18 cases had an AF sample available for AFGGT testing; after the exclusion of cases with abnormalities that might affect the AFGGT level, we included nine cases in the analysis. Because there was only one case of BA in our cohort and 11 more were described in the literature (Table 3), clinical application of these research findings requires caution, as well as careful pre- and post-test counselling.

In addition to the one case of BA in this cohort, two additional cases of BA were not included in this study because prenatal ultrasound scans did not indicate NVFGB. The AFGGT levels in all three cases were <30 U/L and <2.5th percentile (27, 28, and 29 U/L at 20^{+3}, 21^{+5}, and 21^{+6} weeks, respectively), whereas the AFGGT levels in all samples used to establish our reference range were >30 U/L. Thus, an AFGGT level <30 U/L may be a useful absolute cut-off for the prediction of BA. Notably, all three cases of extrahepatic bile duct obstruction reported by Muller et al[5] also had an AFGGT level <30 U/L (20 U/L for all three cases [<1st percentile]). Further research with a larger cohort is required to confirm the efficacy of using an AFGGT level <30 U/L as the absolute cut-off for predicting BA in NVFGB.

Conclusion

Based on the present findings and published literature, we conclude that AFGGT testing is useful for the exclusion of fetal BA in pregnancies with NVFGB. With a consistent NPV of 100% across all published series, AFGGT level ≥2.5th percentile can provide reassurance for parents that the fetus is unlikely to have BA. However, considering its PPV of 33.3% to 75% before 22 weeks of gestation and 17% to 43% after 22 weeks of gestation, AFGGT

level <2.5th percentile cannot be considered diagnostic for BA. Instead, it serves as a warning sign, indicating the need for prompt postnatal investigation of possible BA. Because NVFGB is also associated with chromosomal abnormalities, amniocentesis is recommended; the advantages of detecting underlying chromosomal abnormalities by CMA and excluding BA through AFGGT testing likely outweigh the 0.3% risk of procedure-related miscarriage.[25] Follow-up prenatal ultrasound scans to visualise the fetal gallbladder should be arranged. Paediatricians should also be alerted for prompt postnatal assessment to facilitate early detection of BA. Timely performance of the Kasai operation can reduce the need for liver transplantation in childhood and improve the rate of overall survival into adulthood to 90%.[26,27]

Author contributions

Concept or design: YH Ting, DS Sahota, FCK Wong, TYT Cheung.
Acquisition of data: TYT Cheung, SR Subramaniam, FCK Wong, MHM Chan, YH Ting, NKL Wong, SL Lau, X Zhu, WT Lui, EKW Chan, YKY Kwok, KW Choy, TY Leung.
Analysis or interpretation of data: TYT Cheung, DS Sahota, FCK Wong, YH Ting, NKL Wong.
Drafting of the manuscript: YH Ting, TYT Cheung, DS Sahota, NKL Wong, FCK Wong, SR Subramaniam.
Critical revision of the manuscript for important intellectual content: DS Sahota, YH Ting, TYT Cheung, FCK Wong, NKL Wong.

All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity.

Conflicts of interest

All authors have disclosed no conflicts of interest.

Funding/support

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Ethics approval

This research was approved by the Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee, Hong Kong (Ref No.: CRE 2020.060). Informed consent for amniocentesis in the current study and storage and use of excess amniotic fluid in future research was obtained from the patients at the time of amniocentesis.

Supplementary material

The supplementary material was provided by the authors and some information may not have been peer reviewed. Accepted supplementary material will be published as submitted by the authors, without any editing or formatting. Any opinions or recommendations discussed are solely those of the authors and are not endorsed by the Hong Kong Academy of Medicine and the Hong Kong Medical Association. The Hong Kong Academy of Medicine and the Hong Kong Medical Association disclaim all liability and responsibility arising from any reliance placed on the content. To view the file, please visit the journal online (https://doi.org/10.12809/hkmj2210567).

References

1. Blazer S, Zimmer EZ, Bronshtein M. Nonvisualization of the fetal gallbladder in early pregnancy: comparison with clinical outcome. Radiology 2002;224:379-82.
2. Di Pasquo E, Kuleva M, Rousseau A, et al. Outcome of non-visualization of fetal gallbladder on second-trimester ultrasound: cohort study and systematic review of literature. Ultrasound Obstet Gynecol 2019;54:582-8.
3. Ting YH, So PL, Cheung KW, Lo TK, Ma TW, Leung TY. Non-visualisation of fetal gallbladder in a Chinese cohort. Hong Kong Med J 2022;28:116-23.
4. Parolini F, Boroni G, Milianti S, et al. Biliary atresia: 20-40-year follow-up with native liver in an Italian centre. J Pediatr Surg 2019;54:1440-4.
5. Muller F, Gauthier F, Laurent J, Schmitt M, Boué J. Amniotic fluid GGT and congenital extrahepatic biliary damage. Lancet 1991;337:232-3.
6. Burc L, Guibourdenche J, Luton D, et al. Establishment of reference values of five amniotic fluid enzymes. Analytical performances of the Hitachi 911. Application to complicated pregnancies. Clin Biochem 2001;34:317-22.
7. Bardin R, Danon D, Tor R, Mashiach R, Vardimon D, Meizner I. Reference values for gamma-glutamyl-transferase in amniotic fluid in normal pregnancies. Prenat Diagn 2009;29:703-6.
8. Dreux S, Boughanim M, Lepinard C, et al. Relationship of non-visualization of the fetal gallbladder and amniotic fluid digestive enzymes analysis to outcome. Prenat Diagn 2012;32:423-6.
9. Bardin R, Ashwal E, Davidov B, Danon D, Shohat M, Meizner I. Nonvisualization of the fetal gallbladder: can levels of gamma-glutamyl transpeptidase in amniotic fluid predict fetal prognosis? Fetal Diagn Ther 2016;39:50-5.
10. Stewart SH, Connors GJ, Hutson A. Ethnicity and gamma-glutamyltransferase in men and women with alcohol use disorders. Alcohol Alcohol 2007;42:24-7.
11. Salomon LJ, Alfirevic Z, Berghella V, et al. Practice guidelines for performance of the routine mid-trimester fetal ultrasound scan. Ultrasound Obstet Gynecol 2011;37:116-26.
12. Hartley JL, Davenport M, Kelly DA. Biliary atresia. Lancet 2009;374:1704-13.
13. Hsiao CH, Chang MH, Chen HL, et al. Universal screening for biliary atresia using an infant stool color card in Taiwan. Hepatology 2008;47:1233-40.
14. Zhan J, Chen Y, Wong KK. How to evaluate diagnosis and management of biliary atresia in the era of liver transplantation in China. World J Paediatr Surg 2018;1:e000002.
15. Chalouhi GE, Muller F, Dreux S, Ville Y, Chardot C. Prenatal non-visualization of fetal gallbladder: beware of biliary atresia! Ultrasound Obstet Gynecol 2011;38:237-8.
16. Rahamtalla D, Al Rawahi Y, Jawa ZM, Wali Y. Cystic biliary atresia in a neonate with antenatally detected abdominal cyst. BMJ Case Rep 2022;15:e246081.
17. Lin AE, Ticho BS, Houde K, Westgate MN, Holmes LB. Heterotaxy: associated conditions and hospital-based prevalence in newborns. Genet Med 2000;2:157-72.

18. Gottschalk I, Stressig R, Ritgen J, et al. Extracardiac anomalies in prenatally diagnosed heterotaxy syndrome. Ultrasound Obstet Gynecol 2016;47:443-9.
19. Ehret W. Use of Anticoagulants in Diagnostic Laboratory Investigations. Geneva: World Health Organization; 1999.
20. Czerkiewicz I, Dreux S, Beckmezian A, et al. Biochemical amniotic fluid pattern for prenatal diagnosis of esophageal atresia. Pediatr Res 2011;70:199-202.
21. Muller C, Czerkiewicz I, Guimiot F, et al. Specific biochemical amniotic fluid pattern of fetal isolated esophageal atresia. Pediatr Res 2013;74:601-5.
22. The Fetal Medicine Foundation. Duodenal atresia. Available from: https://fetalmedicine.org/education/fetal-abnormalities/gastrointestinal-tract/duodenal-atresia. Accessed 19 Jan 2023.
23. Ochoa JH, Chiesa M, Vildoza RP, Wong AE, Sepulveda W. Evaluation of the perianal muscular complex in the prenatal diagnosis of anorectal atresia in a high-risk population. Ultrasound Obstet Gynecol 2012;39:521-7.
24. Chen M, Leung TY, Sahota DS, et al. Ultrasound screening for fetal structural abnormalities performed by trained midwives in the second trimester in a low-risk population—an appraisal. Acta Obstet Gynecol Scand 2009;88:713-9.
25. Salomon LJ, Sotiriadis A, Wulff CB, Odibo A, Akolekar R. Risk of miscarriage following amniocentesis or chorionic villus sampling: systematic review of literature and updated meta-analysis. Ultrasound Obstet Gynecol 2019;54:442-51.
26. Serinet MO, Wildhaber BE, Broué P, et al. Impact of age at Kasai operation on its results in late childhood and adolescence: a rational basis for biliary atresia screening. Pediatrics 2009;123:1280-6.
27. Davenport M. Biliary atresia: clinical aspects. Semin Pediatr Surg 2012;21:175-84.