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Supplementary material

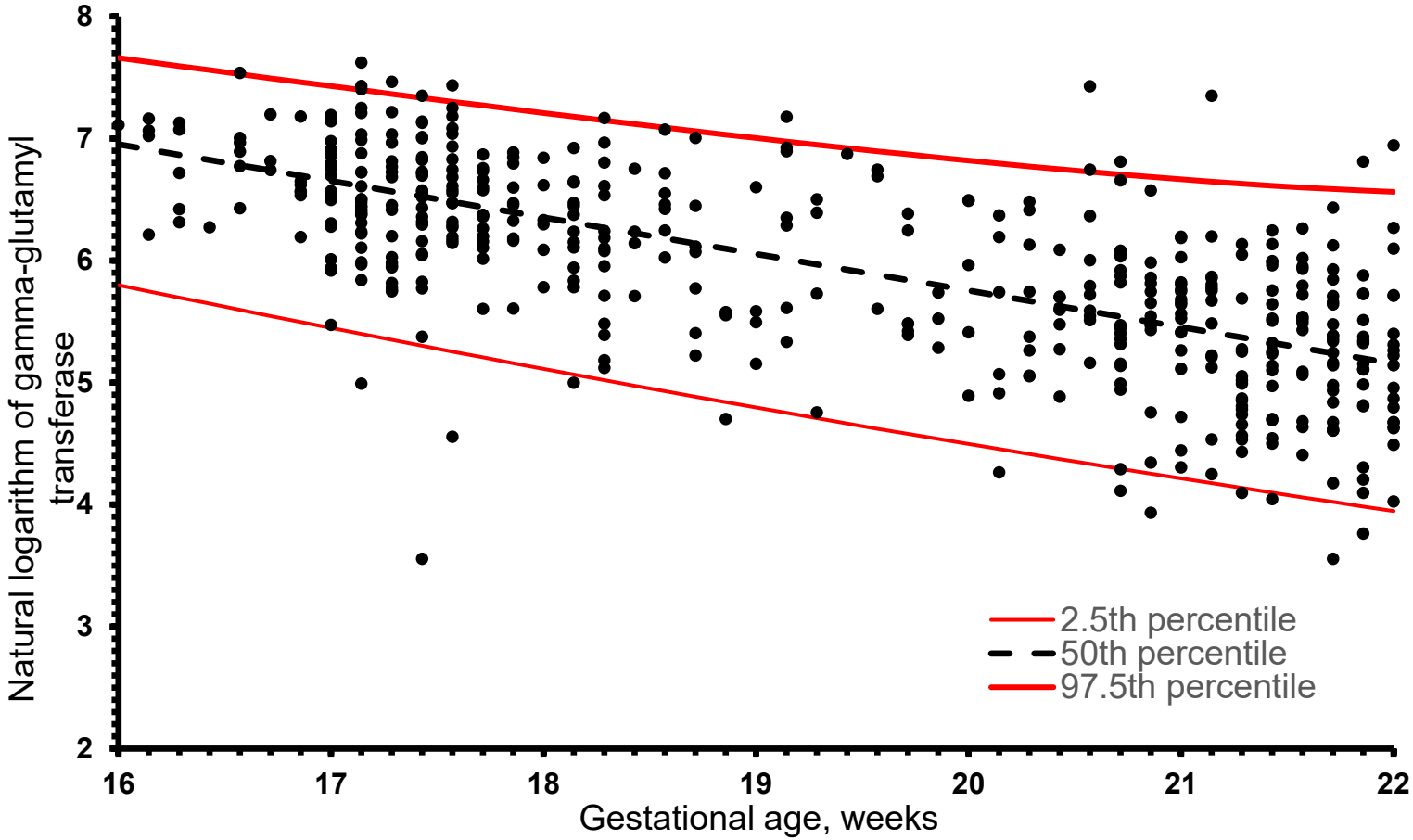
Supplement to: TYT Cheung, NKL Wong, DS Sahota, et al. Amniotic fluid gamma-glutamyl transpeptidase for prediction of biliary atresia in cases of non-visualisation of the fetal gallbladder: a retrospective study using a validated analytical platform and local reference range. Hong Kong Med J 2024 Jun;30(3):218-26 | Epub 5 Jun 2024. https://doi.org/10.12809/hkmj2210567.

Supplementary Table. Regressed values of amniotic fluid gamma-glutamyl transpeptidase levels (in U/L) according to percentile from 16^{+0} to 22^{+6} weeks of gestation

Gestational age, weeks	2.5th percentile	5th percentile	50th percentile	95th percentile	97.5th percentile
16	330	422	1045	1922	2121
16^{+1}	313	401	1001	1856	2051
16^{+2}	298	381	959	1793	1984
16^{+3}	283	363	919	1731	1919
16^{+4}	269	345	880	1673	1857
16^{+5}	256	329	844	1616	1797
16^{+6}	244	313	808	1562	1739
17	232	298	774	1509	1684
17^{+1}	221	283	742	1459	1630
17^{+2}	210	270	711	1411	1579
17^{+3}	201	257	681	1364	1530
17^{+4}	191	245	653	1319	1482
17^{+5}	182	233	625	1276	1437
17^{+6}	174	223	599	1235	1393
18	166	212	574	1195	1351
18^{+1}	158	202	550	1157	1311
18^{+2}	151	193	527	1120	1272
18^{+3}	145	184	505	1085	1235
18^{+4}	138	176	484	1051	1199
18^{+5}	132	168	463	1018	1165
18^{+6}	126	160	444	987	1132
19	121	153	425	957	1100
19^{+1}	116	146	408	928	1070
19^{+2}	111	140	391	900	1041
19^{+3}	106	133	374	874	1013
19^{+4}	102	128	358	848	987
19^{+5}	97	122	343	824	962
19^{+6}	93	117	329	800	938
20	90	112	315	778	915
20^{+1}	86	107	302	757	893
20^{+2}	83	102	289	736	872
20^{+3}	79	98	277	716	853
20^{+4}	76	94	266	698	834

20^{+5}	73	90	255	680	816
20^{+6}	70	86	244	663	800
21	68	83	234	647	785
21^{+1}	65	79	224	631	770
21^{+2}	63	76	215	617	757
21^{+3}	60	73	206	603	745
21^{+4}	58	70	197	590	734
21^{+5}	56	67	189	578	724
21^{+6}	54	65	181	567	715
22	52	62	173	557	708
22^{+1}	50	60	166	547	702
22^{+2}	48	57	159	538	697
22^{+3}	46	55	152	530	694
22^{+4}	45	53	146	523	693
22^{+5}	43	51	140	517	693
22^{+6}	42	49	134	512	695

Supplementary Figure. Reference curves of natural log-transformed amniotic fluid gamma-glutamyl transpeptidase values for the 2.5th, 50th, and 97.5th percentiles at 16^{+0} to 22^{+6} weeks of gestation



Appendix 1. Validation and analytical performance evaluation of amniotic fluid gamma-glutamyl transpeptidase assay

I. Sample preparation

Samples were thawed and equilibrated to room temperature immediately before analysis. Gamma-glutamyl transpeptidase (GGT) activity in samples was assayed with the 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, [city and country]). Internal quality controls were performed before and after each batch of samples. Internal quality control failure was defined using Westgard rules 1_{3s} and 2_{2s}.

II. Validation and analytical performance evaluation

To evaluate the analytical performance of the assay using amniotic fluid, tests were conducted regarding matrix effects; linearity; precision; carryover; and interference due to haemolysis, icterus, and lipaemia. In tests of matrix effects and interference, acceptable recovery was defined as a mean GGT activity recovery of 90% to 110%.

Matrix effects and linearity

Pooled amniotic fluid samples with high GGT level (>1000 U/L; 10% by volume) were spiked into pooled plasma samples (90% by volume) and recovery was calculated. Additionally, pooled GGT-amniotic fluid samples were diluted with 9% sodium chloride (analyser's on-board diluent for GGT), and measured results were compared with expected values. Non-linearity was detected by inspection of the difference plots.

Precision

Intra-run and inter-run precision were tested by repeated analysis of aliquots of the pooled samples in a single run and 20 runs over 20 days, respectively. Additionally, a pooled sample with the lowest GGT level was tested in quadruplicate over 5 days to verify the limit of quantification.

Interference

Interference due to haemolysis, icterus, and lipaemia was tested by spiking freeze shock-lysed red blood cells, unconjugated bilirubin, or 20% intravenous fat emulsion (10% by volume; diluted with 0.9% sodium chloride) into a pooled amniotic fluid sample (90% by volume) with a GGT level approximating 40 U/L (ie, the level at which the manufacturer performed interference studies for plasma/serum samples, as specified in the package insert). Effects on assay accuracy and precision were evaluated. Three

samples at each interferent level were tested to determine the means and distributions of GGT recovery from the samples.

Sample stability

Sample stability was assessed by storing amniotic fluid samples with various GGT concentrations in plain bottles at room temperature, 4℃, -20℃, and -80℃; samples were re-tested after 1 month and 6 months of storage. Acceptable stability was arbitrarily defined as 90% to 110% GGT activity after storage compared with baseline.

Appendix 2. Analytical performance of amniotic fluid gamma-glutamyl transpeptidase assay

I. Analytical performance

Assay performance using amniotic fluid was generally comparable to its specifications for use with plasma or serum.

Matrix effects and linearity

Spiking of plasma samples with high gamma-glutamyl transpeptidase (GGT) levels into pooled amniotic fluid samples with low GGT levels yielded mean (± standard deviation) recovery of 104% (± 9.4%). One outlier was detected; the mean recovery was 101% (± 3.7%) after exclusion of the outlier (online supplementary Appendix Table 1).

Appendix Table 1. Recovery in spiking-and-recovery experiment. Plasma samples with high gamma-glutamyl transpeptidase (GGT) levels were spiked into pooled amniotic fluid samples with low GGT levels, and recovery was calculated. Overall mean (± standard deviation) recovery was 104% (± 9.35%); it was 101% (± 3.69%) when the single outlier was excluded

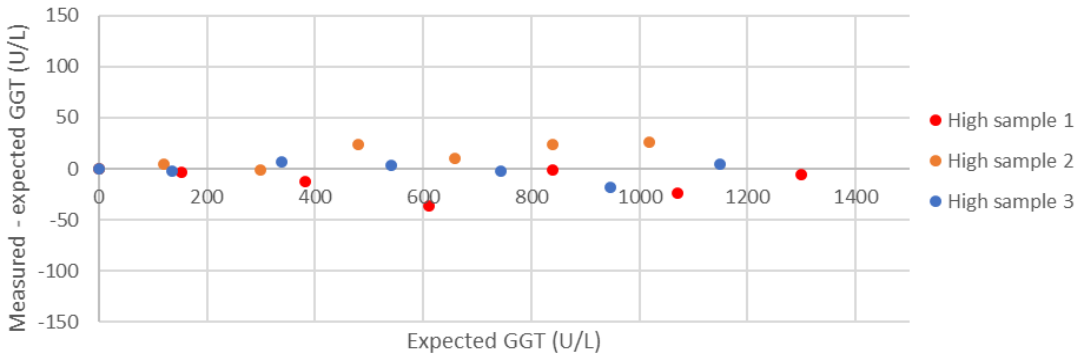
GGT in:				
Plasma (10% by volume), U/L	**Amniotic fluid (90% by volume), U/L**	**Expected, U/L**	**Measured, U/L**	**Recovery**
1833	26	207	226	109%
1833	43	222	229	101%
1833	22	203	214	105%
1345	26	158	160	100%
1345	43	173	171	95%
1345	22	154	155	99%
1080	26	131	168	131%
1080	43	147	153	102%
1080	22	128	135	105%
2995	26	323	330	102%
2995	43	338	343	100%
2995	22	319	319	99%
Outlier included:				Mean ± SD:
				104% ± 9.35%
Outlier excluded:				Mean ± SD:
				101% ± 3.69%

Abbreviation: SD = standard deviation

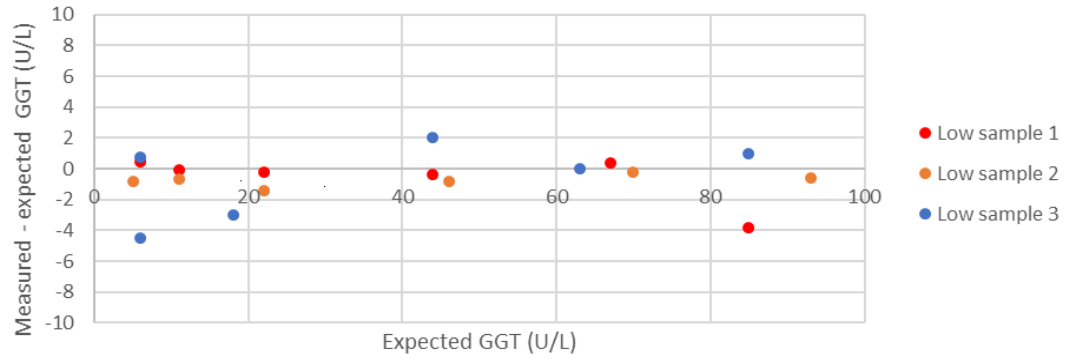
No non-linearity was detected within the GGT range of 6 to 1200 U/L after amniotic fluid samples had been diluted with 9% sodium chloride. Overall, these results indicate the absence of matrix effects on the measurement of GGT in amniotic fluid (online supplementary Appendix Fig 1).

Appendix Figure 1. Serial dilution linearity. Samples with low (a) and high (b) gamma-glutamyl transpeptidase (GGT) levels were diluted with 9% sodium chloride. No non-linearity was detected

(a)



(b)



Precision

Intra- and inter-run precision results were similar to specifications (online supplementary Appendix Table 2). Intra-run precision values at GGT levels of 18, 95, and 770 U/L were 2.1%, 0.9%, and 0.6%, respectively. Inter-run precision values at GGT levels of 18, 95, and 770 U/L were 5.2%, 1.8%, and 2.1%, respectively. Additionally, the limit of quantification was verified at 10 U/L using an actual amniotic fluid sample, with an intermediate coefficient of variation of 6.0% (online

supplementary Appendix Table 3). This limit of quantification is considerably below the 2.5th percentile at each gestational age from 16 to 22 weeks. The 2.5th percentile is the proposed cut-off for excluding a diagnosis of biliary atresia (see below). Therefore, the verified analytical measurement range (10-1200 U/L) was considered sufficient for clinical use.

Appendix Table 2. Analytical precision. Intra-run and inter-run precision were tested at three gamma-glutamyl transpeptidase levels. Results were comparable to specifications for serum and plasma

	Intra-run precision–GGT (U/L) in			**Inter-run precision–GGT (U/L) in**		
	Low sample	**Middle sample**	**High sample**	**Low sample**	**Middle sample**	**High sample**
	18	95	772	18	95	787
	18	94	771	18	96	787
	17	95	769	18	96	760
	18	94	769	18	92	773
	18	95	768	18	96	780
	18	96	772	18	93	760
	18	96	772	20	94	761
	18	94	768	18	94	751
	18	96	776	18	96	800
	18	94	774	18	96	749
	18	96	773	18	97	792
	18	94	766	17	97	790
	17	95	775	19	93	784
	18	96	779	18	93	768
	17	96	778	21	94	769
	18	96	776	18	96	783
	18	96	774	19	94	819
	18	95	762	17	97	771
	18	95	779	17	94	790
	18	94	778	18	93	812
Mean ± SD, U/L	17.9 ± 0.366	95.1 ± 0.852	772 ± 4.59	18.2 ± 0.951	94.9 ± 1.66	779 ± 16.4
CV	2.05%	0.896%	0.595%	5.23%	1.75%	2.11%

Abbreviations: CV = coefficient of variation; GGT = gamma-glutamyl transpeptidase; SD = standard deviation

Appendix Table 3. Verification of limit of quantification. The limit of quantification was verified at 10 U/L, with a coefficient of variation below 20%

	GGT, U/L
	10
	10
	9
	10
	10
	10
	9
	10
	10
	11
	10
	10
	10
	10
	11
	9
	10
	10
	11
	11
Mean ± SD, U/L	10.1 ± 0.605
Coefficient of variation	6.02%

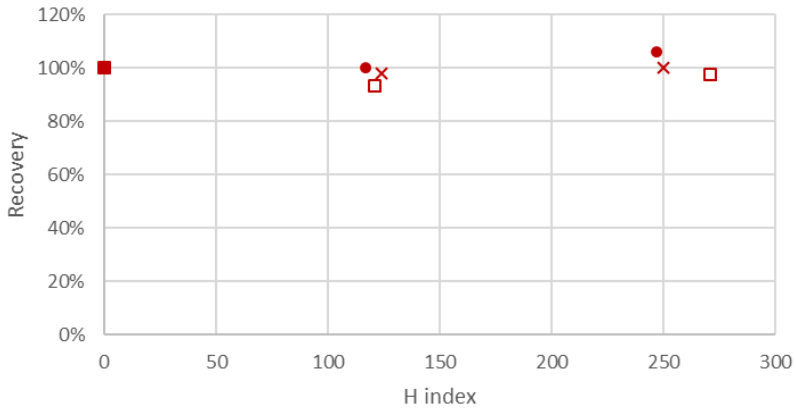
Abbreviation: GGT = gamma-glutamyl transpeptidase; SD = standard deviation

Interference

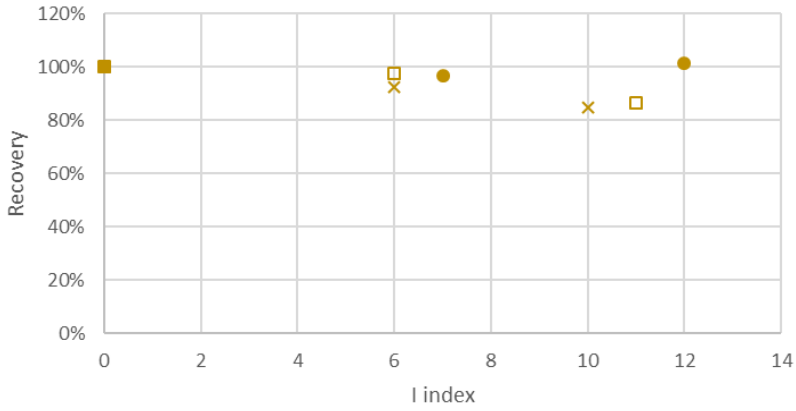
The results of the interference experiment are summarised in online supplementary Appendix Figure 2. Interferents were spiked into a pooled sample, yielding haemolysis (H) indices ≤250, icterus (I) indices ≤12, and lipemia (L) indices ≤600. No significant interference was observed at an H index of 250, an I index of 6, or an L index of 600; these indices correspond to 0.25 g/dL haemoglobin, 103 μmol/L bilirubin, and 16.8 mmol/L triglyceride, respectively. The limits of haemolysis/icterus/lipemia indices above which interference occurred were significantly higher than degrees of those indices in all analysed samples (>700).

Appendix Figure 2. Effects of haemolysis (a), icterus (b), and lipemia (c) on gamma-glutamyl transpeptidase measurement. No significant interference was observed at an H index ≤250, an I index ≤12, or L index ≤600 (recovery within 100% ± 10%)

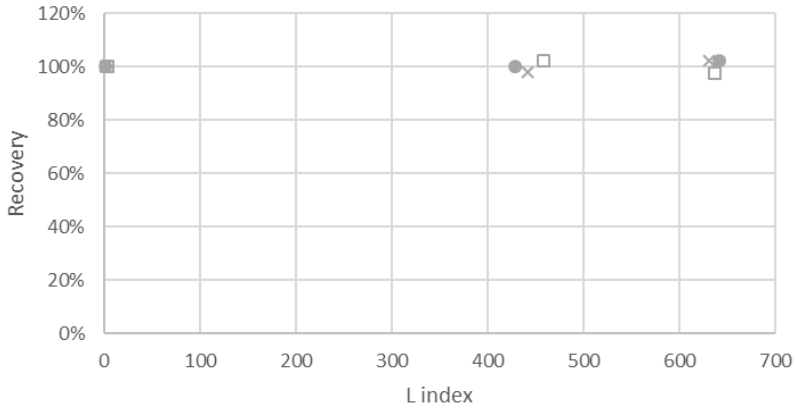
(a)



(b)



(c)



II. Sample stability

Acceptable recovery was demonstrated in all samples stored at room temperature or

4℃ for 1 month, and in all samples stored at -20℃ or -80℃ for 6 months (online supplementary Appendix Table 4).

Appendix Table 4. Sample stability

Sample ID	Baseline GGT, U/L	Temperature	GGT after 1 month, U/L	GGT after 6 months, U/L	Recovery after 1 month	Recovery after 6 months
RT-1	69	RT	68	67	98.6%	97.1%
RT-2	140	RT	135	137	96.4%	97.9%
RT-3	305	RT	295	271	96.7%	88.9%*
RT-4	632	RT	636	191	100.6%	30.2%*
RT-5	765	RT	749	783	97.9%	102.4%
4C-1	28	4℃	29	26	103.6%	92.9%
4C-2	171	4℃	163	158	95.3%	92.4%
4C-3	216	4℃	209	194	96.8%	89.8%*
4C-4	489	4℃	485	492	99.2%	100.6%
4C-5	638	4℃	640	606	100.3%	95.0%
4C-6	931	4℃	890	926	95.6%	99.5%
-20C-1	80	-20℃		83		103.8%
-20C-2	226	-20℃		232		102.7%
-20C-3	467	-20℃		458		98.1%
-20C-4	513	-20℃		516		100.6%
-20C-5	663	-20℃		652		98.3%
-80C-1	90	-80℃		91		101.1%
-80C-2	99	-80℃		95		96.0%
-80C-3	130	-80℃		130		100.0%
-80C-4	213	-80℃		211		99.1%

-80C-5	455	-80°C	454	99.8%
-80C-6	534	-80°C	515	96.4%
-80C-7	765	-80°C	760	99.3%
-80C-8	962	-80°C	952	99.0%

Abbreviations: GGT = gamma-glutamyl transpeptidase; RT = room temperature

* Results outside the predefined 90% to 110% acceptability limits