BMJ Open

A prospective, cross-sectional study protocol to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: The HAPPI Kids study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-025897
Article Type:	Protocol
Date Submitted by the Author:	10-Aug-2018
Complete List of Authors:	Hoq, Monsurul ; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne, Department of Paediatrics Karlaftis, Vicky; Murdoch Children's Research Institute, Haematology Research Mathews, Susan; The Royal Children's Hospital, Department of Biochemistry, Laboratory Services Burgess, Janet; The Royal Children's Hospital, Department of Pathology Collection, Laboratory Services Donath, Susan; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne , Department of Paediatrics Carlin, John; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne , Department of Paediatrics Monagle, Paul; Murdoch Children's Research Institute, Haematology Research; The University of Melbourne , Department of Paediatrics Ignjatovic, Vera; Murdoch Children's Research Institute, Haematology Research; The University of Melbourne, Department of Paediatrics
Keywords:	PAEDIATRICS, Neonates, Children, Reference Intervals

A prospective, cross-sectional study protocol to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: The HAPPI Kids study

Corresponding Author

Associate Professor Vera Ignjatovic, PhD, B.Sc. (Hons)

Co-Group Leader, Haematology Research, Murdoch Children's Research Institute

Principal Fellow, Department of Paediatrics, The University of Melbourne

Address

Haematology Research, Murdoch Children's Research Institute

The Royal Children's Hospital

50 Flemington Road, Parkville, Victoria, 3052, Australia

Tel: +61 3 99366520, Email: vera.ignjatovic@mcri.edu.au

Co-authors

Monsurul Hoq

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: monsurul.hoq@mcri.edu.au

Vicky Karlaftis

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia.

Email: vasiliki.karlaftis@mcri.edu.au

Sue Mathews

Department of Biochemistry, Laboratory Services, The Royal Children's Hospital, Australia.

Email: Susan.Matthews@rch.org.au

Janet Burgess

Department of Pathology Collection, Laboratory Services, The Royal Children's Hospital, Parkville, Australia.

Email: Janet.Burgess@rch.org.au

Susan Donath

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute; Parkville, Australia; Department of Paediatrics, The University of Melbourne; Parkville, Australia.

Email: Susan.donath@mcri.edu.au

John Carlin

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute, Parville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: john.carlin@mcri.edu.au

Paul Monagle

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: Paul.Monagle@rch.org.au

Vera Ignjatovic

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: vera.ignjatovic@mcri.edu.au

WORD COUNT: 2792

ABSTRACT

Introduction:

The clinical interpretation of laboratory tests is reliant on reference intervals. However, the accuracy of a reference interval are dependent on the selected reference population, and in paediatrics, the ability of the reference interval to reflect changes associated with growth, age, sex, and ethnicity. Differences in reagent formulations, methodologies, and analyser can also impact on a reference interval. To date, no direct comparison of reference intervals for common analytes using different analysers in children have been published. The Harmonizing Age Pathology Parameters in Kids (HAPPI Kids) study aims to establish age-appropriate reference intervals for commonly used analytes in the routine clinical care of neonates and children, and to determine the feasibility of paediatric reference interval harmonisation by comparing age-appropriate, multiple analytes reference intervals derived by different analysers.

Methods and analysis:

The HAPPI Kids study is a prospective cross-sectional study, collecting paediatric blood samples for analysis of commonly requested biochemical, immunological and haematological tests. Venous blood samples are collected from healthy premature neonates (32 to 36 weeks), term neonates (from birth to up to a maximum of 72 hours post-birth), and children aged 30 days to 18 years (undergoing minor day surgical procedures). Blood samples are processed according to standard laboratory procedures and, if not processed immediately, stored at -80^{0}C. A minimum of 20 samples is analysed for every analyte for neonates and then each year of age until 18 years. Analytical testing is performed according to the standard operating procedures used for clinical samples. Where possible, sample aliquots from the same patients are analysed for an analyte across multiple commercially available analysers.

Ethics and dissemination:

The study protocol was approved by The Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (34183 A). The study findings will be published in peer-reviewed journals and shared with clinicians, laboratory-scientists and laboratories.

Keywords: paediatrics, neonates, children, reference intervals

STRENGTHS AND LIMITATIONS OF THIS STUDY

1. The prospective design of the study ensures blood samples are collected from healthy neonates and children aged 30 days to 18 years.
2. Aliquots from the same blood sample are tested for common analytes on multiple commercially available analysers allowing direct head to head comparison.
3. A minimum of 20 samples is analysed for neonates and then each year of age until 18 years with equal numbers of males and females (total 380 samples per analyte).
4. Parametric statistical methods are to be applied for estimating reference intervals that vary continuously with increasing age.
5. Changes associated with age are based on population changes not individual longitudinal testing.

INTRODUCTION

Reference intervals are important for the correct interpretation of clinical laboratory tests and impact the clinical assessment and care of patients. Reference intervals are used for determining normal versus abnormal for a measurement of interest, and are usually expressed as a range encompassing 95% of the reference population, where lower and upper limits are 2.5th and 97.5th centile respectively [1].

Obtaining accurate data from a suitable reference population is an essential element in determining reference intervals, and therefore the characteristics of a reference population need to be matched to a “normal” or a healthy population using strict criteria. However, collecting blood samples from children for different laboratory tests is challenging due to the small blood volume that can be safely collected from children [2]. Previously documented paediatric reference interval studies are frequently based on retrospective analysis of laboratory data, prospective sample collection from school children in the community (ie limited age groups) or from paediatric hospitals where children are undergoing minor elective surgery [3-7]. Very few of these studies have included neonates through to 18 years of age [8].

In children, development and growth are among the key challenges in determining reference intervals for different analytes [2]. The dynamic nature of different analytes as children grow and develop influence the representation of paediatric reference intervals, which have often historically been established or partitioned for different age-ranges [8, 9]. This approach can often lead to

misdiagnosis, especially when the age of the child that is being investigated is in close proximity to the borders of the partitions. For example, a child aged three years and 360 days may have clinical results that would be classified as outside the reference interval of 0 to 4 years for a blood test of interest, but within reference interval for children aged 4 to 9 years. Hence, the difference of 5 days results in the test result being labelled as abnormal. Misdiagnosis leads to repeated blood tests for the child, increased utilisation of health services, and is often associated with anxiety for parents and children alike. The determination of the age-specific partition points has often been arbitrary and often there is not an even distribution of the number of reference subjects included across different partitions. For these and other reasons, the process of determining age-specific reference intervals for different analytes based on the artificial partitioning of age has been found to be problematic [4]. A possible solution to address the age-related changes in analyte is to constructing age-appropriate reference intervals considering age as a continuous variable [1, 4, 10].

The laboratory test results for most analytes are dependent on the analyser and reagents used for analysis and therefore, analyser and reagent specific reference intervals should be determined. In several studies, mathematical algorithms or "transference" mechanisms have been applied to compare and construct reference intervals for different analysers based on the reference values for one specific analyser [11, 12]. However, to date, direct comparison of the reference intervals for common analytes using different analysers has not been undertaken in neonates and children.

The Harmonizing Age Pathology Parameters in Kids (HAPPI Kids) study is designed to address the existing gaps in reference interval studies with prospectively collected blood samples from healthy neonates and children, from birth until 18 years of age.

METHODS AND ANALYSIS

The HAPPI Kids study aims

- Establish age-appropriate reference intervals for commonly used analytes in clinical practice in the setting of neonates and children.
- Determine whether analyser specific differences alter age-appropriate reference intervals for multiple analytes in order to determine whether paediatric reference intervals can be harmonised.

Study design

The HAPPI Kids study is a prospective cross-sectional study for the collection of paediatric blood samples for commonly requested biochemical, immunological and haematological analysis.

Study subjects

Healthy premature neonates (32 to 36 weeks), term neonates (from birth up to a maximum of 72 hours post-birth and children aged 30 days to 18 years (undergoing minor day surgical procedures) are recruited based on specific and detailed eligibility criteria in table 1.

Table 1: Summary of the inclusion and exclusion criteria at screening

	Inclusion criteria	Exclusion criteria (any one)
Age groups		
Premature neonates	• Gestational age of 32 to 36 weeks • Generally healthy	• Presence of systemic abnormalities • Requires interpreter
Term neonates	• From birth up to the maximum 72 hours post-birth • Gestational age ≥37 weeks • Weight ≥ 2500 grams • APGAR Score ≥7 at five minutes	
Paediatric	• Attending hospital for minor elective surgery requiring general anaesthetic (30 days to 18 years) OR volunteer to participate in the study after seeing a flyer related to the study (15 to 18 years)	
Category		
Haematology		• Presence of coagulation disorders • Family history of coagulation disorders • Currently on anticoagulation medication
Immunology		• Presence of immune system disorder or immune deficiency syndrome • Presence of genetic disorder • Presence of rheumatologic disorder • Family history of rheumatologic disorder or immune deficiency syndrome • Infection or a febrile illness within the last 7 days • Hospital admission for intravenous (IV) antibiotics to

		clear an infection on more than 2 occasions in life • Has needed 2 or more months of oral antibiotics more than 2 occasions in their life • Failure to thrive • Recipient of blood products in the last 3 months
Biochemistry		• History of liver and renal disease • Presence of endocrine diseases • Presence of metabolic disease • Presence of renal disease • Presence of hepatic disease • Failure to thrive

Participant recruitment and sample collection:

Participant recruitment commenced in February 2015 in four major public hospitals in Melbourne, Australia. Parents or guardians of eligible study participants are approached by Pathology Collection staff at each site and written consent is obtained following a verbal and written explanation of the study and participation requirements. Following patient consent, the Pathology Collection staff interview the parents or guardian using the study questionnaire to confirm study inclusion and exclusion criteria status (table 1) and to assess and document the child's general health.

Three distinct routes of patient recruitment are utilised:

1. Pre-term neonatal samples are collected from "healthy" neonates, born at 32 to 36 weeks, gestation at The Royal Women's Hospital. Samples are collected via direct venepuncture, using a 23-gauge needle into required blood collection tubes. The pre-term neonates do not have any systemic abnormalities, abnormal foetal monitoring and do not require any mechanical ventilation.

2. Term neonatal samples are collected from "healthy" term neonates following routine intramuscular administration of one milligram of vitamin K in the delivery suites at The Royal Women's Hospital, Northern Health and Western Health - Sunshine Hospital. Samples are collected from birth up to a maximum of 72 hours post-birth in hospital via direct venepuncture, using a 23-gauge needle into the required blood collection tubes (Sarstedt). To minimise the number of blood collection procedures performed, the venous sample is also utilised for application to the routine newborn screening Guthrie card.

3. Blood samples from healthy children aged 30 days to 18 years are collected at The Royal Children's Hospital, Northern Hospital and Western Health - Sunshine Hospital. The samples are obtained from "healthy" children prior to minor elective day surgery (e.g. circumcision). Other than elective surgery, these children are deemed "healthy" and are not receiving any medications. All children are fasted before their elective surgery.

Only one attempt to collect blood per participant, per sample is made during the HAPPI Kids study. Blood samples are collected into specific tubes required by the analyser manufacturer depending on the analytes being tested (e.g. serum vs citrate). The participant recruitment and sample collection are currently on going.

Sample sizes

A minimum of 20 samples is collected for 19 specific age groups e.g. neonates and every year of life from 30 days to 18 years[13]. For the biochemical and haematological parameters, a total of 380 samples are analysed. For the immunological analysis, the number of age groups is increased from 19 to 22 (i.e. 0 to 2 months, 2 to 6 months, 6 to 9 months, 9 to 12 months, 12 to 18 months, 18 to 24 months, and every year of life from 3 years to 18 years), equating to a total of 440 samples. For pre-term neonates, a total of 100 samples are collected to facilitate analysis of 20 samples for each week of gestation from 32 to 36 weeks.

The sample size of 380 or 440 for estimating 95% reference intervals of an analyte using parametric methods with an even spread across age is considered adequate based on the approach proposed by Royston [1]. Using a formula for the approximate standard error of the reference limit at the mean value of age, he suggested 292 as a suitable sample size for constructing a 95% reference intervals based on restricting the standard error of the limits of the resulting reference intervals to be no more than 10 percent of the residual standard deviation from the parametric model [1].

Sample processing and storage

The blood samples are processed and stored according to the standard procedures based on the parameters appropriate for the tests being conducted on that sample (e.g. fresh vs frozen).

For biochemistry analytes, samples are collected into S-Monovette serum gel tubes (Sarstedt), centrifuged at 5000rpm and 6 °C for 5 minutes, separated and stored in 400 uL aliquots at -80 °C within four hours of collection.

For immunology analytes, samples are collected into either S-Monovette serum gel tubes (Sarstedt), or S-Monovette neutral tubes (Sarstedt), depending on the downstream tests. The samples are spun

at 5000rpm and 6 °C for 5 minutes, separated and the resultant serum stored in 400uL aliquots at -80 °C within four hours of collection.

For haematology analytes, samples are collected into S-Monovette citrate 3.2% tubes (Sarstedt). The samples are centrifuged at 3800rpm at room temperature for 5 minutes, and plasma is stored in 500uL aliquots at -80 °C within four hours of collection, using a previously established collection protocol [14].

Samples utilised for determination of blood groups (ABO) and the thalassemia screen are collected into 500uL S-Monovette EDTA tubes and processed in the laboratory within 24 hours of collection.

Cell count samples are collected in S-Monovette EDTA tubes (Sarstedt) and processed by the laboratory within 3 hours of collection.

Sample testing

Blood samples collected from the study participants are tested according to documented and accredited standard operating procedures used for the testing of patient samples within the participating laboratories. The biochemical, immunological and haematological analytes tested are listed in table 2. Where indicated (*), sample aliquots from the same patient are tested for the same analyte using different automated analysers commonly in clinical use to facilitate direct head to head comparison. In addition, where indicated in table 2 ($^{+}$), aliquots of the same sample are tested in different laboratories using the same analyser type and test method. The sample testing is currently ongoing.

Table 2: List of analytes tested for the HAPPI Kids study.

Analytes			
Biochemical	**Immunological**	**Haematological**	**Pre-term**
Sodium*$^{+}$	Immunoglobulin G*	Factors: XII, XI, IX, X, II, VII, VIII, V	Urea Electrolytes and Creatinine
Potassium*$^{+}$	Immunoglobulin A*	Inhibitors: Protein C, Protein S, Antithrombin, Alpha-2-macroglobulin	liver function tests
Chloride*$^{+}$	Immunoglobulin M*	Von Willebrand factor	Calcium
Bicarbonate*$^{+}$	Rheumatoid factor*	Collegen binding assay	Magnesium
Urea*$^{+}$	Complement component 3*	Ristocetin cofactor assay	Phosphate
Creatinine *$^{+}$	Complement component 4*	Activated Partial Thromboplastin Time	Ferritin
Total Bilirubin	Cystatin C*	Prothrombin Time	Activated Partial

			Thromboplastin Time
Conjugated Bilirubin*+	ANTI-STREPTOLYSIN O TEST*	Fibrinogen	Prothrombin Time
Alkaline Phosphate*+	Thyroid peroxidase*	Thrombin clotting time	Fibrinogen
Aspartate Aminotransferase *+	Thyroglobulin*	D-Dimers	D-Dimers
Alanine Aminotransferase *+	Iron*	Full Blood Examination and Reticulocytes	
Gamma-Glutamyl Transferase *+	Ferritin*	Red Cell Folate	
Total Protein*+	Transferrin*	Glucose-6-phosphate dehydrogenase	
Albumin*+	Antinuclear antibody	Active B12	
Calcium*+	Soluble FAS Ligand	Total Homocysteine	
Magnesium*+	Soluble CD25	Total B12	
Phosphate*+	Immunoglobulin E	Serum Folate	
Lactate dehydrogenase*+	Classic Haemolytic Complement Pathway		
Creatine Kinase *+	Alternative Haemolytic Complement Pathway		
Lipase*+	Mannose-binding lectin Complement Pathway		
Amylase*+			
Uric Acid*+			
Triglycerides *+			
Cholesterol*+			
High-density lipoprotein*+			
Thyroid Stimulating Hormone *+			
Free Thyroxine *+			
Free Triiodothyronine *+			
Anti-Müllerian hormone *			
Oestradiol*			
Sex hormone binding globulin *			
Dehydroepiandrosterone – Sulphate*			
Cortisol*			
Growth Hormone*			
Testosterone*			
25-hydroxyvitamin D*			
High Sensitivity			

Oestradiol*			
Androstenedione*			
17α-Hydroxyprogesterone *			
Insulin-like growth factor 1*			
Insulin-like growth factor-binding protein-3 *			

* Sample aliquots from the same patient are tested for the same analyte using different automated analysers in common clinical use to facilitate direct head to head comparison.

+ Aliquots of the same sample are tested in different laboratories using the same analyser type and test method.

Data management system

Study data is collected and managed using the REDCap (Research Electronic Data Capture) electronic data capture tools hosted at the Murdoch Children's Research Institute [15]. REDCap is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

Data analysis

Test results for different analytes will be plotted according to age in order to identify outliers, explore the normality assumption, and to assess the association between analyte and age-specific variability. Following a detailed examination, including assessment of biological implausibility, outliers will to be excluded from analysis. Reference intervals will be constructed using parametric statistical methods in two steps. In the first step, a fractional polynomial regression model of age will be fitted to the mean of normally distributed or log transformed test results of an analyte, using sex as a covariate[16]. In the second steps, the 2.5^{th} and 97.5^{th} centiles will be estimated using quantile regression where the power variables of age from the fractional polynomial regression model and potential interaction with sex are used as covariates [17]. The 95% confidence interval of the reference intervals will be estimated based on the fitted model of the reference limits. A combination of statistical testing, i.e. goodness of fit and variance component analysis, and clinical expertise will be used to determine the extent to which the reference intervals of an analyte based on different analysers can be compared.

Study timeframe

The participant recruitment and sample collection commenced on February 2015 and is expected to be completed by August 2021. The testing of samples for 30 biochemical analytes has commenced, while testing of samples for the remaining biochemical analytes and immunological and haematological analytes are currently on going. We plan to complete the testing of sample by February 2021.

Patient and public involvement

The study was conceived in response to patient complaints about having blood tests done at community pathology laboratories and their children requiring repeat testing because age appropriate normal data was not available. The recruitment and consent processes were piloted and parental/ child feedback incorporated into the main study protocol. A summary of the results will be shared by mail to all the participants involved. If any child has a clinically abnormal result the parents are contacted and appropriate plans for review of the child are made.

ETHICS AND DISSEMINATION

The study protocol has been approved (34183 A) by The Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (HREC) and subsequently approved by the HREC committees of all participating hospitals including, The Royal Women's Hospital, Northern Health and Western Health - Sunshine Hospital. The study is primarily supported by The Royal Children's Hospital Foundation and is based at the Haematology Research Laboratory, Murdoch Children's Research Institute, Melbourne, Australia.

Written consent is obtained from parents or guardians of eligible study participants following a verbal and written explanation of the study and participation requirements by Pathology Collection staff at each site after. Less than 3% of the blood volume is collected from a child considering the amount of blood volume a child has, while only one attempt is made to minimise risk associated with collecting intravenous blood.

Participant's identifying information is replaced by a study number (ID) at recruitment to maintain confidentiality. This ID has been used in all laboratory specimens, evaluation forms, reports and other records that leaves the site. Electronic data are stored in REDCap which can only be accessed by the authorised members of the research team. Similarly, paper-based information are stored in a locked filing cabinet and can only be accessed by the designated persons. The blood samples are stored in freezers which are only accessible to authorised members of the research team by using their individual swipe card maintaining a record of who has accessed the samples and at what time.

All results which will fall outside the final calculated reference range will be reviewed by the investigators in conjunction with discipline specific advisors. In the cases where the reviewing group believes that the result is of clinical significance, the families are going to be contacted and the blood tests are going to be repeated. If necessary, an appropriate referral will be made and the individual will be followed up by the appropriate specialist.

At the conclusion of the study, blood samples collected as part of this study will be destroyed, unless consent has been given for them to be stored in a biobank located at the Haematology Research Laboratory for use in future research.

The study results will be summarised for submission to peer-reviewed journals. Results will be shared with participating hospitals and laboratories and presented at local meetings, national and international conferences.

DISCUSSION

Reference intervals are used on a daily basis by clinicians to interpret measurements obtained from patients [18]. The accuracy and reliability of the reference intervals are highly dependent on the reference population utilised to define those reference intervals [11, 19]. Harris and Boyed recommend that characteristics of the sample from the reference population should be similar to those of the patients *[20].* Paediatric reference intervals established by laboratories using retrospective data mining techniques provide extensive sample numbers. However, the representativeness of the population is often compromised by using the laboratory results of children who were initially referred for a significant clinical investigation [8]. Samples collected prospectively from pre-schools are useful in constructing reference intervals, but are applicable to pre-school aged children only[6]. Addressing the issue Canadian Initiative in Paediatric Reference Intervals (CALIPER) and German Health Interview and Examination Survey for Children and Diagnosis (KiGGS) collected data prospectively using community-based approach ensuring representativeness [10, 21, 22]. Similarly, the HAPPI Kids study is one of the very few studies collecting data prospectively from pre-defined healthy preterm neonates and term neonates from birth until 72 hours post birth, as well as children from 30 days to 18 years of age.

Samples collected for HAPPI Kids utilise paediatric Pathology Collectors with specific skills and documented competency to collect venous samples from neonates. All collected samples meet RCH laboratory standard operating procedures for collection and storage as documented in the Specimen Collection Handbook (SCH) for the analytes [23]. All participating laboratories perform daily quality control, participated in external quality assurance programs (Royal College of Pathologists of

Australasia Quality Assurance Programs (RCPA QAP)) and were accredited by the National Association of Testing Authorities (NATA). The prospective design of the HAPPI Kids study also ensures quality of sample collection, storage and testing in different laboratories.

The direct comparison of reference intervals by analysers is not possible using data mining techniques as the blood sample available might not be enough to test in all available analysers. To overcome this problem CALIPER program which initially established reference intervals using the Abbott ARCHITECT analyser transferred reference intervals in line with Clinical & Laboratory Standards Institute (CLSI) recommendations by testing only 100 samples on Beckman Coulter DxC800, Ortho Vitros 5600, Roche Cobas 600 and Siemens Vista 1500 using an algorithm [11, 12]. The study used $r^2 < 0.70$ as an indicator of non-transference between Abbott ARCHITECT and another platform and reported few analytes as non-transferable between analysers[11]. The challenges in transference from Abbott ARCHITECT to another analyser were discussed by the authors including the lack of an appropriate number of samples per age group since only 100 samples were tested to predict the linear relationship [11]. The HAPPI Kids study utilises aliquots of the same blood sample from the same patient and tests the aliquots on different analysers, enabling the first documented head to head comparison between analysers for the age-spectrum covering neonates to children 18 years of age. Therefore, results of the HAPPI Kids study will be very useful in improving interpretation of pathology results for neonates and children.

REFERENCE

1. Royston, P., *Constructing time-specific reference ranges.* Statistics in Medicine, 1991. **10**(5): p. 675-690.
2. Coffin, C.M., et al., *Pediatric laboratory medicine: current challenges and future opportunities.* American Journal Of Clinical Pathology, 2002. **117**(5): p. 683-690.
3. Adeli, K., *Closing the gaps in pediatric reference intervals: The CALIPER initiative.* Clinical Biochemistry, 2011. **44**(7): p. 480-482.
4. Loh, T.P., et al., *Development of paediatric biochemistry centile charts as a complement to laboratory reference intervals.* Pathology, 2014. **46**(4): p. 336-43.
5. *Pediatric reference intervals: Critical gap analysis and establishment of a national initiative.* Clinical Biochemistry, 2006. **39**(6): p. 559-560.
6. Southcott, E.K., et al., *Establishment of pediatric reference intervals on a large cohort of healthy children.* Clinica Chimica Acta, 2010. **411**(19–20): p. 1421-1427.
7. Shaw, J.L.V., et al., *Validity of establishing pediatric reference intervals based on hospital patient data: A comparison of the modified Hoffmann approach to CALIPER reference intervals obtained in healthy children.* Clinical Biochemistry, 2014. **47**(3): p. 166-172.
8. Colantonio, D.A., et al., *Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children.* Clin Chem, 2012. **58**(5): p. 854-68.
9. Daly, C.H., et al., *A systematic review of statistical methods used in constructing pediatric reference intervals.* Clinical Biochemistry, 2013. **46**(13–14): p. 1220-1227.

10. Kohse, K.P., *KiGGS — The German survey on children's health as data base for reference intervals and beyond.* Clinical Biochemistry, 2014. **47**(9): p. 742-743.
11. Estey, M.P., et al., *CLSI-based transference of the CALIPER database of pediatric reference intervals from Abbott to Beckman, Ortho, Roche and Siemens Clinical Chemistry Assays: Direct validation using reference samples from the CALIPER cohort.* Clinical Biochemistry, 2013. **46**(13–14): p. 1197-1219.
12. CLSI, *CLSI Releases Guidelines for Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory*. 2008, COMTEX News Network, Inc.
13. Ignjatovic, V., et al., *Age-related differences in heparin response.* Thrombosis Research, 2006. **118**(6): p. 741-745.
14. Monagle, P., et al., *Developmental haemostasis. Impact for clinical haemostasis laboratories.* Thromb Haemost, 2006. **95**(2): p. 362-72.
15. Harris, P.A., et al., *Research Electronic Data Capture (REDCap) - A metadata-driven methodology and workflow process for providing translational research informatics support.* Journal of biomedical informatics, 2009. **42**(2): p. 377-381.
16. Royston, P. and E.M. Wright, *A method for estimating age-specific reference intervals ('normal ranges') based on fractional polynomials and exponential transformation.* Journal of the Royal Statistical Society: Series A (Statistics in Society), 1998. **161**(1): p. 79-101.
17. Wei, Y., et al., *Quantile regression methods for reference growth charts.* Statistics in Medicine, 2006. **25**(8): p. 1369-1382.
18. Koerbin, G., J.R. Tate, and A.C.f.C.R.I. on behalf of the, *Harmonising Adult Reference Intervals in Australia and New Zealand – the Continuing Story.* The Clinical Biochemist Reviews, 2016. **37**(3): p. 121-129.
19. Ritchie, R.F. and G. Palomaki, *Selecting clinically relevant populations for reference intervals.* Clinical Chemistry And Laboratory Medicine, 2004. **42**(7): p. 702-709.
20. Forsman, R., *Statistical Bases of Reference Values in Laboratory Medicine. Eugene K. Harris and James C. Boyd. New York; Marcel Dekker, Inc., 1995. Hardback $165.00. ISBN 0-8247-9339-0.* Clinical Chemistry, 1996. **42**(8): p. 1303.
21. Adeli, K., et al., *The Canadian laboratory initiative on pediatric reference intervals: A CALIPER white paper.* Crit Rev Clin Lab Sci, 2017. **54**(6): p. 358-413.
22. Ridefelt, P., *Pediatric reference intervals - the Swedish experience.* Clin Biochem, 2014. **47**(9): p. 740-1.
23. Melbourne, T.R.C.s.H. *RCH Specimen collection handbook*. Available from: https://ww2.rch.org.au/specimen/.

AUTHOR STATEMENT

MH wrote the manuscript, and developed the statistical analysis plan. VK wrote the study protocol, obtained ethics approval for the study, co-wrote the manuscript and is the co-ordinator of the study. SM provided Biochemistry expertise in the design of the study and revised the manuscript. JB provided pathology collection expertise in the design of the study, training for the pathology collectors and reviewed the manuscript. JC and SD provided support for statistical analysis plan and reviewed the manuscript. PM conceived the study and contributed to its design and was a major contributor in writing the manuscript. VI contributed to the design of the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

FUNDING

This study has been primarily funded by The Royal Children's Hospital Foundation (Proposal ID: 2014 – 233), with supplementary funding from Ortho Diagnostics. In addition, the study received in-kind reagents from Roche Diagnostics. Funding bodies do not have any input in the design of the study, sample collection, analysis and interpretation of data and in preparation of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

The authors are grateful to the parents and children for their participation in this study. The authors thank members of the Pathology Collection Department of The Royal Children's Hospital for the consent of participants and collection of samples. The authors acknowledge the contribution of the members of the anaesthetic and surgical departments of the Royal Children's Hospital, Northern Hospital and Western Health - Sunshine Hospital, Melbourne, for facilitation of sample collection. In addition, the authors thank members of the post-natal departments of The Royal Women's Hospital, Northern Hospital and Western Health, Sunshine Hospital.

BMJ Open

A prospective, cross-sectional study to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: The HAPPI Kids study

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-025897.R1
Article Type:	Protocol
Date Submitted by the Author:	14-Jan-2019
Complete List of Authors:	Hoq, Monsurul ; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne, Department of Paediatrics Karlaftis, Vicky; Murdoch Children's Research Institute, Haematology Research Mathews, Susan; The Royal Children's Hospital, Department of Biochemistry, Laboratory Services Burgess, Janet; The Royal Children's Hospital, Department of Pathology Collection, Laboratory Services Donath, Susan; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne , Department of Paediatrics Carlin, John; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne , Department of Paediatrics Monagle, Paul; Murdoch Children's Research Institute, Haematology Research; The University of Melbourne , Department of Paediatrics Ignjatovic, Vera; Murdoch Children's Research Institute, Haematology Research; The University of Melbourne, Department of Paediatrics
Primary Subject Heading:	Paediatrics
Secondary Subject Heading:	Paediatrics
Keywords:	PAEDIATRICS, Neonates, Children, Reference Intervals

A prospective, cross-sectional study to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: The HAPPI Kids study

Corresponding Author

Associate Professor Vera Ignjatovic, PhD, B.Sc. (Hons)

Co-Group Leader, Haematology Research, Murdoch Children's Research Institute

Principal Fellow, Department of Paediatrics, The University of Melbourne

Address

Haematology Research, Murdoch Children's Research Institute

The Royal Children's Hospital

50 Flemington Road, Parkville, Victoria, 3052, Australia

Tel: +61 3 99366520, Email: vera.ignjatovic@mcri.edu.au

Co-authors

Monsurul Hoq

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: monsurul.hoq@mcri.edu.au

Vicky Karlaftis

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia.

Email: vasiliki.karlaftis@mcri.edu.au

Sue Mathews

Department of Biochemistry, Laboratory Services, The Royal Children's Hospital, Australia.

Email: Susan.Matthews@rch.org.au

Janet Burgess

Department of Pathology Collection, Laboratory Services, The Royal Children's Hospital, Parkville, Australia.

Email: Janet.Burgess@rch.org.au

Susan Donath

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute; Parkville, Australia; Department of Paediatrics, The University of Melbourne; Parkville, Australia.

Email: Susan.donath@mcri.edu.au

John Carlin

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: john.carlin@mcri.edu.au

Paul Monagle

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: Paul.Monagle@rch.org.au

Vera Ignjatovic

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: vera.ignjatovic@mcri.edu.au

WORD COUNT: 3746

ABSTRACT

Introduction:

The clinical interpretation of laboratory tests is reliant on reference intervals. However, the accuracy of a reference interval is dependent on the selected reference population, and in paediatrics, the ability of the reference interval to reflect changes associated with growth and age, as well as sex and ethnicity. Differences in reagent formulations, methodologies, and analysers can also impact on a reference interval. To date, no direct comparison of reference intervals for common analytes using different analysers in children has been published. The HAPPI Kids study aims to establish age-appropriate reference intervals for commonly used analytes in the routine clinical care of neonates and children, and to determine the feasibility of paediatric reference interval harmonisation by comparing age-appropriate reference intervals in different analysers for multiple analytes.

Methods and analysis:

The HAPPI Kids study is a prospective cross-sectional study, collecting paediatric blood samples for analysis of commonly requested biochemical, immunological and haematological tests. Venous blood samples are collected from healthy premature neonates (32 to 36 weeks gestation), term neonates (from birth to a maximum of 72 hours post-birth), and children aged 30 days to ≤ 18 years (undergoing minor day surgical procedures). Blood samples are processed according to standard laboratory procedures and, if not processed immediately, stored at – 80^0C. A minimum of 20 samples is analysed for every analyte for neonates and then each year of age until 18 years. Analytical testing is performed according to the standard operating procedures used for clinical samples. Where possible, sample aliquots from the same patients are analysed for an analyte across multiple commercially available analysers.

Ethics and dissemination:

The study protocol was approved by The Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (34183 A). The study findings will be published in peer-reviewed journals and shared with clinicians, laboratory-scientists and laboratories.

Keywords: paediatrics, neonates, children, reference intervals

STRENGTHS AND LIMITATIONS OF THIS STUDY

1. The prospective design of the study ensures blood samples are collected from healthy neonates and children aged 30 days to ≤ 18 years.
2. Aliquots from the same blood sample are tested for common analytes on multiple commercially available analysers allowing direct head to head comparison.
3. Samples are obtained from a minimum of 20 neonates and then for 20 children within each year of age until 18 years with equal numbers of males and females (total 380 samples per analyte).
4. Parametric statistical methods are to be applied for estimating reference intervals that vary continuously with increasing age.
5. Changes in analytes associated with age are based on samples collected cross-sectionally not based on individuals followed longitudinally.

INTRODUCTION

Reference intervals are important for the correct interpretation of clinical laboratory tests and affect the clinical assessment and care of patients. Reference intervals are used for determining normal versus abnormal for a measurement of interest, and are usually defined as a range encompassing 95% of the reference population, where lower and upper limits are 2.5th and 97.5th centile respectively [1].

Obtaining accurate data from a suitable reference population is an essential element in determining reference intervals, and therefore the characteristics of a reference population need to be matched to a “normal” or a healthy population using strict criteria. However, collecting blood samples from children for different laboratory tests is challenging due to the small blood volume that can be safely collected from children and the ethical concern of obtaining blood from healthy children [2]. For these reasons, previously documented paediatric reference interval studies are frequently based on retrospective analysis of laboratory data, prospective sample collection from school children in the community (i.e. limited age groups) or from paediatric hospitals where children are undergoing minor elective surgery [3-7]. Regardless of how the samples are collected, very few of these studies have included neonates through to 18 years of age [8].

In children, development and growth are among the key challenges in determining reference intervals for different analytes [2]. The dynamic nature of different analytes as children grow and develop influences the representation of paediatric reference intervals, which have often historically been

established or partitioned for different age-ranges [8, 9]. This approach can often lead to misdiagnosis, especially when the age of the child that is being investigated is in close proximity to the borders of the partitions. For example, a child aged three years and 360 days may have clinical results that would be classified as outside the reference interval of 0 to 4 years for a blood test of interest, but within the reference interval for children aged 4 to 9 years. Hence, the difference of 5 days results in the test result being labelled as abnormal. Misdiagnosis leads to repeated blood tests for the child, increased utilisation of health services, and is often associated with anxiety for parents and children alike. The determination of the age-specific partition points has often been arbitrary and often there is not an even distribution of the number of reference subjects included across different partitions. For these and other reasons, the process of determining age-specific reference intervals for different analytes based on the artificial partitioning of age has been found to be problematic [4]. A preferred approach, which addresses the age-related changes in analyte levels, is to construct age-appropriate reference intervals considering age as a continuous variable [1, 4, 10].

The laboratory test results for most analytes are dependent on the analyser and reagents used for analysis and therefore, analyser- and reagent-specific reference intervals should be determined. In several studies, mathematical algorithms or "transference" mechanisms have been applied to compare and construct reference intervals for different analysers based on the reference values for one specific analyser [11, 12]. However, to date, direct comparison of the reference intervals for common analytes using different analysers has not been undertaken in neonates and children.

The Harmonizing Age Pathology Parameters in Kids (HAPPI Kids) study is designed to address the existing gaps in reference interval studies with prospectively collected blood samples from healthy neonates and children, from birth until 18 years of age.

METHODS AND ANALYSIS

The HAPPI Kids study: Aims

- Establish age-appropriate reference intervals for commonly used analytes in clinical practice in the setting of neonates and children.
- Determine whether analyser characteristics alter age-appropriate reference intervals for multiple analytes, in order to determine whether paediatric reference intervals can be harmonised.

Study design

The HAPPI Kids study is a prospective cross-sectional study for the collection of paediatric blood samples for commonly requested biochemical, immunological and haematological analysis.

Study subjects

The study subjects consist of healthy premature neonates (32 to 36 weeks gestation), term neonates (from birth up to a maximum of 72 hours post-birth and children aged 30 days to 18 years (undergoing minor day surgical procedures).

Participant recruitment and sample collection:

Participant recruitment commenced in February 2015 in four major public hospitals in Melbourne, Australia. Parents or guardians of eligible study participants are approached by Pathology Collection staff at each site and written consent is obtained following a verbal and written explanation of the study and participation requirements. Following patient consent, the Pathology Collection staff interview the parents or guardian using the study questionnaire (supplementary table 1) to confirm study inclusion and exclusion criteria status (table 1*).* In addition, the child's medical record is reviewed to assess and document the child's general health.

Three distinct routes of patient recruitment are utilised:

1. Pre-term neonatal samples are collected from "healthy" neonates about to be discharged from post-natal wards, born at 32 to 36 weeks gestation, at The Royal Women's Hospital. Samples are collected in the first three days of life via direct venepuncture, using a 23-gauge needle, into required blood collection tubes. The pre-term neonates do not have any systemic abnormalities i.e. underlying diseases and comorbidilites, abnormal foetal monitoring and do not require any mechanical ventilation.
2. Term neonatal samples are collected from "healthy" term neonates about to be discharged from post-natal wards following routine intramuscular administration of one milligram of vitamin K in the delivery suites at The Royal Women's Hospital, Northern Health and Western Health - Sunshine Hospital. Samples are collected from birth up to a maximum of 72 hours post-birth in hospital via direct venepuncture, using a 23-gauge needle into the required blood collection tubes (Sarstedt). To minimise the number of blood collection procedures performed, the venous sample is also utilised for application to the routine newborn screening Guthrie card.

3. Blood samples from healthy children aged 30 days to 18 years are collected at The Royal Children's Hospital, Northern Hospital and Western Health - Sunshine Hospital. The samples are obtained from "healthy" children prior to minor elective day surgery (e.g. circumcision). Other than elective surgery, these children are deemed "healthy" and are not receiving any medications. All children are fasted before their elective surgery.

Only one attempt to collect blood per participant, per sample is made during the HAPPI Kids study. Blood samples are collected into specific tubes required by the analyser manufacturer depending on the analytes being tested (e.g. serum vs citrate). The participant recruitment and sample collection are currently on going.

Table 1: Summary of the inclusion and exclusion criteria at screening

	Inclusion criteria	Exclusion criteria (any one)
Age groups		
Premature neonates	• Gestational age of 32 to 36 weeks • Generally healthy	• Presence of systemic abnormalities • Requires interpreter
Term neonates	• From birth up to the maximum 72 hours post-birth • Gestational age ≥37 weeks • Weight ≥ 2500 grams • APGAR Score ≥7 at five minutes	
Paediatric	• Attending hospital for minor elective surgery requiring general anaesthetic (30 days to 18 years) OR volunteer to participate in the study after seeing a flyer related to the study (15 to 18 years)	
Category		
Haematology		• Presence of coagulation disorders • Family history of coagulation disorders • Currently on anticoagulation medication
Immunology		• Presence of immune system disorder or immune deficiency syndrome • Presence of genetic disorder

		• Presence of rheumatologic disorder • Family history of rheumatologic disorder or immune deficiency syndrome • Infection or a febrile illness within the last 7 days • Infection or a febrile illness within the las 4 weeks • Hospital admission for intravenous (IV) antibiotics to clear an infection on more than 2 occasions in life • Has needed 2 or more months of oral antibiotics more than 2 occasions in their life • Failure to thrive • Recipient of blood products in the last 3 months • Diagnosed with food allergy, asthma, eczema or hayfever • Family history of food allergy, asthma, eczema or food allergy
Biochemistry		• History of liver and renal disease • Presence of endocrine diseases • Presence of metabolic disease • Presence of renal disease • Presence of hepatic disease • Failure to thrive

Sample size

A minimum of 20 samples is collected for 19 specific age groups e.g. neonates and every year of life from 30 days to 18 years [1]. For the biochemical and haematological parameters, a total of 380 samples are analysed. For the immunological analysis, the number of age groups is increased from 19 to 22 (i.e. 1 to 2 months, 2 to 6 months, 6 to 9 months, 9 to 12 months, 12 to 18 months, 18 to 24 months, and every year of life from 3 years to 18 years), equating to a total of 440 samples. For pre-term neonates, a total of 100 samples are collected to facilitate analysis of 20 samples for each week of gestation from 32 to 36 weeks.

The sample size of 380 or 440 for estimating 95% reference intervals of an analyte using parametric methods with samples distributed uniformly across 30 days to < 18 years is considered adequate based on the approach proposed by Royston [1]. Using a formula for the approximate standard error of the reference limit at the mean value of age, he suggested 292 as a suitable sample size for

constructing a 95% reference intervals based on restricting the standard error of the limits of the resulting reference intervals to be no more than 10 percent of the residual standard deviation from the parametric model [1].

Sample processing and storage

Samples are thawed as per standard clinical laboratory practice for the analyte in question. There are no repeat freeze-thaw cycles for any of the samples. For some analytes the samples are processed immediately and not frozen, within the same time frame and conditions under which clinical samples would be handled. Analytes that can be frozen will be tested in batches. Testing will be done with samples from all ages included in each batch with randomly assigned orders of testing to avoid any batch to batch bias.

For biochemistry analytes, samples are collected into S-Monovette serum gel tubes (Sarstedt), centrifuged at 5000rpm and 6 °C for 5 minutes, separated and stored in 400 µL aliquots at -80 °C within four hours of collection.

For immunology analytes, samples are collected into either S-Monovette serum gel tubes (Sarstedt), or S-Monovette neutral tubes (Sarstedt), depending on the downstream tests. The samples are spun at 5000rpm and 6 °C for 5 minutes, separated and the resultant serum stored in 400µL aliquots at -80 °C within four hours of collection.

For haematology analytes, samples are collected into S-Monovette citrate 3.2% tubes (Sarstedt). The samples are centrifuged at 3800rpm at room temperature for 5 minutes, and plasma is stored in 500µL aliquots at -80 °C within four hours of collection, using a previously established collection protocol [13].

Samples utilised for determination of blood groups (ABO) and the thalassemia screen are collected into 500uL S-Monovette EDTA tubes and processed in the laboratory within 24 hours of collection.

Cell count samples are collected in S-Monovette EDTA tubes (Sarstedt) and processed by the laboratory within 3 hours of collection.

The details of blood tubes used in sample collection is provided in table 2.

Table 2: Blood tubes used in sample collection

Analytes catogy	Tube Type	Manufacturer	Subtype	Volume	Product Code	Age group

Biochemistry	SST	Sarstedt	S-Monovette	7.5 mL	01.1602.001	Neonate and Paediatric
Haematology	EDTA	Sarstedt	S-Monovette	2.7 mL	05.1167.001	Paediatric
	EDTA	Sarstedt	Micro Tube	0.5 mL	41.1395.002	Neonate
	Sodium Citrate	Sarstedt	S-Monovette	3 mL	05.1165.100	Paediatric
	Sodium Citrate	Sarstedt	S-Monovette	1.4 mL	06.1668.100	Neonate
	Lithium Heparin	Sarstedt	S-Monovette	7.5 mL	01.1608.001	Paediatric
	Lithium Heparin	Sarstedt		0.5 mL	20.1345	Neonate
Immunology	SST	Sarstedt	S-Monovette	7.5 mL	01.1602.001	Neonate and Paediatric
	Neutral	Sarstedt	S-Monovette	7.5 mL	01.1728.001	Neonate and Paediatric

Sample testing

Blood samples collected from the study participants are tested according to documented and accredited standard operating procedures used for the testing of patient samples within the participating laboratories. The biochemical, immunological and haematological analytes tested are listed in table 3. Where indicated (*), sample aliquots from the same patient are tested for the same analyte using different automated analysers commonly in clinical use to facilitate direct head to head comparison. In addition, where indicated in table 3 (†), aliquots of the same sample are tested in different laboratories using the same analyser type and test method. The sample testing is currently ongoing.

Table 3: List of analytes tested for the HAPPI Kids study.

Biochemical	Immunological	Haematological
Sodium*††	Immunoglobulin G*	Factors: XII, XI, IX, X, II, VII, VIII, V
Potassium*††	Immunoglobulin A*	Inhibitors: Protein C, Protein S, Antithrombin, Alpha-2-macroglobulin
Chloride*††	Immunoglobulin M*	Von Willebrand factor
Bicarbonate*††	Rheumatoid factor*	Collegen binding assay

Urea*+†	Complement component 3*	Ristocetin cofactor assay
Creatinine *+†	Complement component 4*	Activated Partial Thromboplastin Time†
Total Bilirubin†	Cystatin C*	Prothrombin Time†
Conjugated Bilirubin*+†	ANTI-STREPTOLYSIN O TEST*	Fibrinogen†
Alkaline Phosphate*+†	Thyroid peroxidase*	Thrombin clotting time
Aspartate Aminotransferase *+†	Thyroglobulin*	D-Dimers†
Alanine Aminotransferase *+†	Iron*	Full Blood Examination and Reticulocytes
Gamma-Glutamyl Transferase *+†	Ferritin*†	Red Cell Folate
Total Protein*+	Transferrin*	Glucose-6-phosphate dehydrogenase
Albumin*+	Antinuclear antibody	Active B12
Calcium*+†	Soluble FAS Ligand	Total Homocysteine
Magnesium*+†	Soluble CD25	Total B12
Phosphate*+†	Immunoglobulin E	Serum Folate
Lactate dehydrogenase*+	Classic Haemolytic Complement Pathway	
Creatine Kinase *+	Alternative Haemolytic Complement Pathway	
Lipase*+	Mannose-binding lectin Complement Pathway	
Amylase*+		
Uric Acid*+		
Triglycerides *+		
Cholesterol*+		
High-density lipoprotein*+		
Thyroid Stimulating Hormone *+		
Free Thyroxine *+		
Free Triiodothyronine *+		
Anti-Müllerian hormone *		
Oestradiol*		
Sex hormone binding globulin *		
Dehydroepiandrosterone – Sulphate*		
Cortisol*		
Growth Hormone*		
Testosterone*		
25-hydroxyvitamin D*		
High Sensitivity Oestradiol*		
Androstenedione*		
17α-Hydroxyprogesterone *		
Insulin-like growth factor 1*		
Insulin-like growth factor-binding protein-3 *		

* Sample aliquots from the same patient are tested for the same analyte using different automated analysers in common clinical use to facilitate direct head to head comparison.

+ Aliquots of the same sample are tested in different laboratories using the same analyser type and test method.

† Samples from pre term children are tested.

Data management system

Study data are collected and managed using the REDCap (Research Electronic Data Capture) electronic data capture system hosted at the Murdoch Children's Research Institute [14]. REDCap is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

Data analysis

Test results for different analytes will be plotted according to age in order to identify outliers, explore normality of distributions, and to assess the association between analyte level and age. Outliers will to be excluded from analysis following a detailed examination, including visual inspection, appropriate statistical tests e.g. Tukey's test and assessment of biological implausibility e.g. haemolysis index for biochemistry samples. Reference intervals will be constructed using parametric statistical methods in two steps. In the first step, a fractional polynomial regression model of age will be fitted to the mean of normally distributed or log transformed test results of an analyte, using sex as a covariate [15]. In the second step, the 2.5^{th} and 97.5^{th} centiles will be estimated using quantile regression where the power variables of age from the fractional polynomial regression model are used as covariates [16]. Potential interaction with sex will be examined. The 95% confidence interval of the reference intervals will be estimated based on the fitted model for the reference limits. A combination of statistical testing, i.e. goodness of fit and variance component analysis, and clinical expertise will be used to determine the extent to which the reference intervals of an analyte based on different analysers can be compared.

Stata 15 will be used for data analysis [17].

Study timeframe

The participant recruitment and sample collection commenced on February 2015 and is expected to be completed by August 2021. The testing of samples for 30 biochemical analytes has commenced, while testing of samples for the remaining biochemical analytes and immunological and haematological analytes are currently on going. We plan to complete the testing of sample by February 2021.

Patient and public involvement

The study was conceived in response to patient complaints about having blood tests done at community pathology laboratories and their children requiring repeat testing because age-appropriate normal data was not available. The recruitment and consent processes were piloted and parental/ child feedback incorporated into the main study protocol. A summary of the results will be shared by mail with all the participants involved. If any child has a clinically abnormal result the parents are contacted and appropriate plans for review of the child are made.

ETHICS AND DISSEMINATION

The study protocol has been approved (34183 A) by The Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (HREC) and subsequently approved by the HREC committees of all participating hospitals including, The Royal Women's Hospital, Northern Health and Western Health - Sunshine Hospital. The study is primarily supported by The Royal Children's Hospital Foundation and is based at the Haematology Research Laboratory, Murdoch Children's Research Institute, Melbourne, Australia.

Written consent is obtained from parents or guardians of eligible study participants following a verbal and written explanation of the study and participation requirements by Pathology Collection staff at each site after. Less than 3% of the blood volume is collected from a child considering the amount of blood volume a child has, while only one attempt is made to minimise risk associated with collecting intravenous blood.

Participants' identifying information is replaced by a study number (ID) at recruitment to maintain confidentiality. This ID is used in all laboratory specimens, evaluation forms, reports and other records that leaves the site. Electronic data are stored in REDCap which can only be accessed by the authorised members of the research team. Similarly, paper-based information are stored in a locked filing cabinet and can only be accessed by the designated persons. The blood samples are stored in freezers that are only accessible to authorised members of the research team by using their individual swipe card maintaining a record of who has accessed the samples and at what time.

All results that fall outside the existing reference range used by the clinical laboratory are referred immediately to the study coordinator to determine whether there is any clinical significance that requires follow-up for the child. If necessary, families are contacted and the blood tests repeated, or an appropriate referral will be made and the individual will be followed up by the appropriate specialist.

At the conclusion of the study, blood samples collected as part of this study will be destroyed, unless consent has been given for them to be stored in a biobank located at the Haematology Research Laboratory for use in future research.

The study results will be summarised for submission to peer-reviewed journals. Results will be shared with participating hospitals and laboratories and presented at local meetings, national and international conferences.

DISCUSSION

Reference intervals are used on a daily basis by clinicians to interpret measurements obtained from patients [18]. The accuracy and reliability of the reference intervals are highly dependent on the reference population utilised to define those reference intervals [11, 19]. As recommended by Harris and Boyd[], the characteristics of the sample from the reference population should be similar to those of the patients [20]. Paediatric reference intervals established by laboratories using retrospective data mining techniques provide extensive sample numbers. However, the representativeness of the population is often compromised by using the laboratory results of children who were initially referred for a significant clinical investigation [8]. Samples collected prospectively from pre-schools are useful in constructing reference intervals, but are applicable to pre-school aged children only [6]. Addressing this issue, the Canadian Initiative in Paediatric Reference Intervals (CALIPER) and German Health Interview and Examination Survey for Children and Diagnosis (KiGGS) collected data prospectively using a community-based approach ensuring representativeness [10, 21, 22]. Similarly, the HAPPI Kids study is one of the very few studies collecting data prospectively from pre-defined healthy preterm neonates and term neonates from birth until 72 hours post birth, as well as children from 30 days to 18 years of age.

Samples collected for HAPPI Kids utilise paediatric Pathology Collectors with specific skills and documented competency to collect venous samples from neonates. All collected samples meet RCH laboratory standard operating procedures for collection and storage as documented in the Specimen Collection Handbook (SCH) for the analytes [23]. All participating laboratories perform daily quality control, participated in external quality assurance programs (Royal College of Pathologists of Australasia Quality Assurance Programs (RCPA QAP)) and were accredited by the National Association of Testing Authorities (NATA). The prospective design of the HAPPI Kids study also ensures quality of sample collection, storage and testing in different laboratories.

The CLSI recommended sample size for establishing reference intervals for a discrete age group is 120 [12]. However, there is no recommendation for sample size when reference intervals are

constructed for continuous age. Few papers have previously reported continuous reference intervals across childhood and are based on retrospective data mining of samples taken for clinical purposes over many years [4, 24]. No previous studies have collected prospective samples (which were not required for clinical management) from neonates and children specifically screened to be a "well population" for the purpose of developing continuous reference intervals. In that context the exact numbers required remain unknown. Based on Royston's recommendation our numbers seem appropriate [1]. However, it is our intention to do a simulation study to develop better guidance for appropriate numbers of samples required to allow reliable characterisation of age-specific analyte dynamics.

The direct comparison of reference intervals by analysers is not possible using data mining techniques, nor when testing remainder of clinically driven samples (due to sample aliquot limitations). To overcome this problem CALIPER program which initially established reference intervals using the Abbott ARCHITECT analyser transferred reference intervals in line with Clinical & Laboratory Standards Institute (CLSI) recommendations by testing only 100 samples on Beckman Coulter DxC800, Ortho Vitros 5600, Roche Cobas 600 and Siemens Vista 1500 using an algorithm [11, 12]. The study used $r^2 < 0.70$ as an indicator of non-transference between Abbott ARCHITECT and another platform and reported several analytes as non-transferable between analysers[11]. The challenges in transference from the Abbott ARCHITECT to another analyser were discussed by the authors including the lack of an appropriate number of samples per age group since only 100 samples were tested to predict the linear relationship [11]. The HAPPI Kids study utilises aliquots of the same blood sample from the same patient and tests the aliquots on different analysers, enabling the first documented head-to-head comparison between analysers for the age spectrum covering neonates to children 18 years of age. Therefore, we expect the results of the HAPPI Kids study to be very useful in improving interpretation of pathology results for neonates and children.

REFERENCE

1. Royston, P., *Constructing time-specific reference ranges.* Statistics in Medicine, 1991. **10**(5): p. 675-690.
2. Coffin, C.M., et al., *Pediatric laboratory medicine: current challenges and future opportunities.* American Journal Of Clinical Pathology, 2002. **117**(5): p. 683-690.
3. Adeli, K., *Closing the gaps in pediatric reference intervals: The CALIPER initiative.* Clinical Biochemistry, 2011. **44**(7): p. 480-482.
4. Loh, T.P., et al., *Development of paediatric biochemistry centile charts as a complement to laboratory reference intervals.* Pathology, 2014. **46**(4): p. 336-43.
5. *Pediatric reference intervals: Critical gap analysis and establishment of a national initiative.* Clinical Biochemistry, 2006. **39**(6): p. 559-560.
6. Southcott, E.K., et al., *Establishment of pediatric reference intervals on a large cohort of healthy children.* Clinica Chimica Acta, 2010. **411**(19–20): p. 1421-1427.

7. Shaw, J.L.V., et al., *Validity of establishing pediatric reference intervals based on hospital patient data: A comparison of the modified Hoffmann approach to CALIPER reference intervals obtained in healthy children.* Clinical Biochemistry, 2014. **47**(3): p. 166-172.
8. Colantonio, D.A., et al., *Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children.* Clin Chem, 2012. **58**(5): p. 854-68.
9. Daly, C.H., et al., *A systematic review of statistical methods used in constructing pediatric reference intervals.* Clinical Biochemistry, 2013. **46**(13–14): p. 1220-1227.
10. Kohse, K.P., *KiGGS — The German survey on children's health as data base for reference intervals and beyond.* Clinical Biochemistry, 2014. **47**(9): p. 742-743.
11. Estey, M.P., et al., *CLSI-based transference of the CALIPER database of pediatric reference intervals from Abbott to Beckman, Ortho, Roche and Siemens Clinical Chemistry Assays: Direct validation using reference samples from the CALIPER cohort.* Clinical Biochemistry, 2013. **46**(13–14): p. 1197-1219.
12. CLSI, *CLSI Releases Guidelines for Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory*. 2008, COMTEX News Network, Inc.
13. Monagle, P., et al., *Developmental haemostasis. Impact for clinical haemostasis laboratories.* Thromb Haemost, 2006. **95**(2): p. 362-72.
14. Harris, P.A., et al., *Research Electronic Data Capture (REDCap) - A metadata-driven methodology and workflow process for providing translational research informatics support.* Journal of biomedical informatics, 2009. **42**(2): p. 377-381.
15. Royston, P. and E.M. Wright, *A method for estimating age-specific reference intervals ('normal ranges') based on fractional polynomials and exponential transformation.* Journal of the Royal Statistical Society: Series A (Statistics in Society), 1998. **161**(1): p. 79-101.
16. Wei, Y., et al., *Quantile regression methods for reference growth charts.* Statistics in Medicine, 2006. **25**(8): p. 1369-1382.
17. StataCrop. *Stata Statisical Software: Release 15.* 2017. Colleage Station, TX: Stata Crop LP.
18. Koerbin, G., J.R. Tate, and A.C.f.C.R.I. on behalf of the, *Harmonising Adult Reference Intervals in Australia and New Zealand – the Continuing Story.* The Clinical Biochemist Reviews, 2016. **37**(3): p. 121-129.
19. Ritchie, R.F. and G. Palomaki, *Selecting clinically relevant populations for reference intervals.* Clinical Chemistry And Laboratory Medicine, 2004. **42**(7): p. 702-709.
20. Eugene K. H and James C. B,*Statistical Bases of Reference Values in Laboratory Medicine,. New York; Marcel Dekker, Inc., 1995.*
21. Adeli, K., et al., *The Canadian laboratory initiative on pediatric reference intervals: A CALIPER white paper.* Crit Rev Clin Lab Sci, 2017. **54**(6): p. 358-413.
22. Ridefelt, P., *Pediatric reference intervals - the Swedish experience.* Clin Biochem, 2014. **47**(9): p. 740-1.
23. Melbourne, T.R.C.s.H. *RCH Specimen collection handbook*. Available from: https://ww2.rch.org.au/specimen/.
24. Zierk, J., et al., *Age- and Sex-Specific Dynamics in 22 Hematologic and Biochemical Analytes from Birth to Adolescence.* Clin Chem, 2015. **61**(7): p. 964-973.

AUTHOR STATEMENT

MH wrote the manuscript, and developed the statistical analysis plan. VK wrote the study protocol, obtained ethics approval for the study, co-wrote the manuscript and is the co-ordinator of the study. SM provided Biochemistry expertise in the design of the study and revised the manuscript. JB

provided pathology collection expertise in the design of the study, training for the pathology collectors and reviewed the manuscript. JC and SD provided support for statistical analysis plan and reviewed the manuscript. PM conceived the study and contributed to its design and was a major contributor in writing the manuscript. VI contributed to the design of the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

FUNDING

This study has been primarily funded by The Royal Children's Hospital Foundation (Proposal ID: 2014 – 233), with supplementary funding from Ortho Diagnostics. In addition, the study received in-kind reagents from Roche Diagnostics. Funding bodies do not have any input in the design of the study, sample collection, analysis and interpretation of data and in preparation of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

The authors are grateful to the parents and children for their participation in this study. The authors thank members of the Pathology Collection Department of The Royal Children's Hospital for the consent of participants and collection of samples. The authors acknowledge the contribution of the members of the anaesthetic and surgical departments of the Royal Children's Hospital, Northern Hospital and Western Health - Sunshine Hospital, Melbourne, for facilitation of sample collection. In addition, the authors thank members of the post-natal departments of The Royal Women's Hospital, Northern Hospital and Western Health, Sunshine Hospital.

Supplementary table 1: Questionnaire for assessing inclusion and exclusion criteria.

Participant/Category	Questions / Data fields
All Sample	Study ID
	Date of Recruitment
	Site of Recruitment
	Date Sample Collected
	Hospital
	UR
	First Name
	Surname
	Date of Birth
	Sex
	Address
	Ethnicity of Mother
	Ethnicity of Father
	Tube Type Collected and Volume of blood collected
Neonates	Gestation
	Time of Birth
	Day of Bleed
	Nature of Delivery
	Weight
	APGAR Score at 5 minutes
	Feeding Method
	Date/Time of Vitamin K
	Current Medications
Paediatric	Procedure performed
	Current Medications
	Clinical Information
Haematology	Dose the participant have a presence of coagulation disorders?
	Does the participant have a family history of coagulation disorders?
	Is there participant currently on anticoagulation medication?
Immunology	Does the participant have a presence of an immune system disorder or immune deficiency syndrome?
	Does the participant have a presence of a genetic disorder?
	Does the participant have a presence of a rheumatologic disorder?
	Has any family member or relative of your child ever been diagnosed with an immune system disorder or immune deficiency syndrome?
	Has the participant had an infection or a febrile illness within the last 7 days?

	Has the participant had a hospital admission for intravenous (IV) antibiotics to clear an infection on more than 2 occasions in life?
	Has the participant needed 2 or more months of oral antibiotics more than 2 occasions in their life
	Does the participant have a history of a failure to thrive?
	Has the participant been a recipient of blood products in the last 3 months?
	Has a doctor ever diagnosed your child with food allergy, asthma, eczema or hayfever?
	Has anyone in your child's immediate family (that is, your child's parents and/or siblings) ever been diagnosed with food allergy, asthma, eczema or hayfever?
Biochemistry	Does the participant have a history of liver and renal disease?
	Does the participant have a presence of endocrine diseases?
	Does the participant have a presence of metabolic disease?
	Does the participant have a presence of renal disease?
	Does the participant have a presence of hepatic disease?
	Does the participant have a history of failure to thrive?

BMJ Open

A prospective, cross-sectional study to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: The HAPPI Kids study protocol

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2018-025897.R2
Article Type:	Protocol
Date Submitted by the Author:	21-Feb-2019
Complete List of Authors:	Hoq, Monsurul ; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne, Department of Paediatrics Karlaftis, Vicky; Murdoch Children's Research Institute, Haematology Research Mathews, Susan; The Royal Children's Hospital, Department of Biochemistry, Laboratory Services Burgess, Janet; The Royal Children's Hospital, Department of Pathology Collection, Laboratory Services Donath, Susan; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne , Department of Paediatrics Carlin, John; Murdoch Children's Research Institute, Clinical Epidemiology and Biostatistics Unit; The University of Melbourne , Department of Paediatrics Monagle, Paul; Murdoch Children's Research Institute, Haematology Research; The University of Melbourne , Department of Paediatrics Ignjatovic, Vera; Murdoch Children's Research Institute, Haematology Research; The University of Melbourne, Department of Paediatrics
Primary Subject Heading:	Paediatrics
Secondary Subject Heading:	Paediatrics
Keywords:	PAEDIATRICS, Neonates, Children, Reference Intervals

A prospective, cross-sectional study to establish age-specific reference intervals for neonates and children in the setting of clinical biochemistry, immunology and haematology: The HAPPI Kids study protocol

Corresponding Author

Associate Professor Vera Ignjatovic, PhD, B.Sc. (Hons)

Co-Group Leader, Haematology Research, Murdoch Children's Research Institute

Principal Fellow, Department of Paediatrics, The University of Melbourne

Address

Haematology Research, Murdoch Children's Research Institute

The Royal Children's Hospital

50 Flemington Road, Parkville, Victoria, 3052, Australia

Tel: +61 3 99366520, Email: vera.ignjatovic@mcri.edu.au

Co-authors

Monsurul Hoq

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: monsurul.hoq@mcri.edu.au

Vicky Karlaftis

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia.

Email: vasiliki.karlaftis@mcri.edu.au

Sue Mathews

Department of Biochemistry, Laboratory Services, The Royal Children's Hospital, Australia.

Email: Susan.Matthews@rch.org.au

Janet Burgess

Department of Pathology Collection, Laboratory Services, The Royal Children's Hospital, Parkville, Australia.

Email: Janet.Burgess@rch.org.au

Susan Donath

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute; Parkville, Australia; Department of Paediatrics, The University of Melbourne; Parkville, Australia.

Email: Susan.donath@mcri.edu.au

John Carlin

Clinical Epidemiology and Biostatistics Unit, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: john.carlin@mcri.edu.au

Paul Monagle

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: Paul.Monagle@rch.org.au

Vera Ignjatovic

Haematology Research, Murdoch Children's Research Institute, Parkville, Australia; Department of Paediatrics, The University of Melbourne, Parkville, Australia.

Email: vera.ignjatovic@mcri.edu.au

WORD COUNT: 3746

ABSTRACT

Introduction:

The clinical interpretation of laboratory tests is reliant on reference intervals. However, the accuracy of a reference interval is dependent on the selected reference population, and in paediatrics, the ability of the reference interval to reflect changes associated with growth and age, as well as sex and ethnicity. Differences in reagent formulations, methodologies, and analysers can also impact on a reference interval. To date, no direct comparison of reference intervals for common analytes using different analysers in children has been published. The HAPPI Kids study aims to establish age-appropriate reference intervals for commonly used analytes in the routine clinical care of neonates and children, and to determine the feasibility of paediatric reference interval harmonisation by comparing age-appropriate reference intervals in different analysers for multiple analytes.

Methods and analysis:

The HAPPI Kids study is a prospective cross-sectional study, collecting paediatric blood samples for analysis of commonly requested biochemical, immunological and haematological tests. Venous blood samples are collected from healthy premature neonates (32 to 36 weeks gestation), term neonates (from birth to a maximum of 72 hours post-birth), and children aged 30 days to ≤ 18 years (undergoing minor day surgical procedures). Blood samples are processed according to standard laboratory procedures and, if not processed immediately, stored at – 80^0C. A minimum of 20 samples is analysed for every analyte for neonates and then each year of age until 18 years. Analytical testing is performed according to the standard operating procedures used for clinical samples. Where possible, sample aliquots from the same patients are analysed for an analyte across multiple commercially available analysers.

Ethics and dissemination:

The study protocol was approved by The Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (34183 A). The study findings will be published in peer-reviewed journals and shared with clinicians, laboratory-scientists and laboratories.

Keywords: paediatrics, neonates, children, reference intervals

STRENGTHS AND LIMITATIONS OF THIS STUDY

1. The prospective design of the study ensures blood samples are collected from healthy neonates and children aged 30 days to ≤ 18 years.
2. Aliquots from the same blood sample are tested for common analytes on multiple commercially available analysers allowing direct head to head comparison.
3. Samples are obtained from a minimum of 20 neonates and then for 20 children within each year of age until 18 years with equal numbers of males and females (total 380 samples per analyte).
4. Parametric statistical methods are to be applied for estimating reference intervals that vary continuously with increasing age.
5. Changes in analytes associated with age are based on samples collected cross-sectionally not based on individuals followed longitudinally.

INTRODUCTION

Reference intervals are important for the correct interpretation of clinical laboratory tests and affect the clinical assessment and care of patients. Reference intervals are used for determining normal versus abnormal for a measurement of interest, and are usually defined as a range encompassing 95% of the reference population, where lower and upper limits are 2.5th and 97.5th centile respectively [1].

Obtaining accurate data from a suitable reference population is an essential element in determining reference intervals, and therefore the characteristics of a reference population need to be matched to a “normal” or a healthy population using strict criteria. However, collecting blood samples from children for different laboratory tests is challenging due to the small blood volume that can be safely collected from children and the ethical concern of obtaining blood from healthy children [2]. For these reasons, previously documented paediatric reference interval studies are frequently based on retrospective analysis of laboratory data, prospective sample collection from school children in the community (i.e. limited age groups) or from paediatric hospitals where children are undergoing minor elective surgery [3-7]. Regardless of how the samples are collected, very few of these studies have included neonates through to 18 years of age [8].

In children, development and growth are among the key challenges in determining reference intervals for different analytes [2]. The dynamic nature of different analytes as children grow and develop influences the representation of paediatric reference intervals, which have often historically been

established or partitioned for different age-ranges [8, 9]. This approach can often lead to misdiagnosis, especially when the age of the child that is being investigated is in close proximity to the borders of the partitions. For example, a child aged three years and 360 days may have clinical results that would be classified as outside the reference interval of 0 to 4 years for a blood test of interest, but within the reference interval for children aged 4 to 9 years. Hence, the difference of 5 days results in the test result being labelled as abnormal. Misdiagnosis leads to repeated blood tests for the child, increased utilisation of health services, and is often associated with anxiety for parents and children alike. The determination of the age-specific partition points has often been arbitrary and often there is not an even distribution of the number of reference subjects included across different partitions. For these and other reasons, the process of determining age-specific reference intervals for different analytes based on the artificial partitioning of age has been found to be problematic [4]. A preferred approach, which addresses the age-related changes in analyte levels, is to construct age-appropriate reference intervals considering age as a continuous variable [1, 4, 10].

The laboratory test results for most analytes are dependent on the analyser and reagents used for analysis and therefore, analyser- and reagent-specific reference intervals should be determined. In several studies, mathematical algorithms or "transference" mechanisms have been applied to compare and construct reference intervals for different analysers based on the reference values for one specific analyser [11, 12]. However, to date, direct comparison of the reference intervals for common analytes using different analysers has not been undertaken in neonates and children.

The Harmonizing Age Pathology Parameters in Kids (HAPPI Kids) study is designed to address the existing gaps in reference interval studies with prospectively collected blood samples from healthy neonates and children, from birth until 18 years of age.

METHODS AND ANALYSIS

The HAPPI Kids study: Aims

- Establish age-appropriate reference intervals for commonly used analytes in clinical practice in the setting of neonates and children.
- Determine whether analyser characteristics alter age-appropriate reference intervals for multiple analytes, in order to determine whether paediatric reference intervals can be harmonised.

Study design

The HAPPI Kids study is a prospective cross-sectional study for the collection of paediatric blood samples for commonly requested biochemical, immunological and haematological analysis.

Study subjects

The study subjects consist of healthy premature neonates (32 to 36 weeks gestation), term neonates (from birth up to a maximum of 72 hours post-birth and children aged 30 days to 18 years (undergoing minor day surgical procedures).

Participant recruitment and sample collection:

Participant recruitment commenced in February 2015 in four major public hospitals in Melbourne, Australia. Parents or guardians of eligible study participants are approached by Pathology Collection staff at each site and written consent is obtained following a verbal and written explanation of the study and participation requirements. Following patient consent, the Pathology Collection staff interview the parents or guardian using the study questionnaire (supplementary table 1) to confirm study inclusion and exclusion criteria status (table 1*).* In addition, the child's medical record is reviewed to assess and document the child's general health.

Three distinct routes of patient recruitment are utilised:

1. Pre-term neonatal samples are collected from "healthy" neonates about to be discharged from post-natal wards, born at 32 to 36 weeks gestation, at The Royal Women's Hospital. Samples are collected in the first three days of life via direct venepuncture, using a 23-gauge needle, into required blood collection tubes. The pre-term neonates do not have any systemic abnormalities i.e. underlying diseases and comorbidilites, abnormal foetal monitoring and do not require any mechanical ventilation.
2. Term neonatal samples are collected from "healthy" term neonates about to be discharged from post-natal wards following routine intramuscular administration of one milligram of vitamin K in the delivery suites at The Royal Women's Hospital, Northern Health and Western Health - Sunshine Hospital. Samples are collected from birth up to a maximum of 72 hours post-birth in hospital via direct venepuncture, using a 23-gauge needle into the required blood collection tubes (Sarstedt). To minimise the number of blood collection procedures performed, the venous sample is also utilised for application to the routine newborn screening Guthrie card.

3. Blood samples from healthy children aged 30 days to 18 years are collected at The Royal Children's Hospital, Northern Hospital and Western Health - Sunshine Hospital. The samples are obtained from "healthy" children prior to minor elective day surgery (e.g. circumcision). Other than elective surgery, these children are deemed "healthy" and are not receiving any medications. All children are fasted before their elective surgery.

Only one attempt to collect blood per participant, per sample is made during the HAPPI Kids study. Blood samples are collected into specific tubes required by the analyser manufacturer depending on the analytes being tested (e.g. serum vs citrate). The participant recruitment and sample collection are currently on going.

Table 1: Summary of the inclusion and exclusion criteria at screening

	Inclusion criteria	Exclusion criteria (any one)
Age groups		
Premature neonates	• Gestational age of 32 to 36 weeks • Generally healthy	• Presence of systemic abnormalities • Requires interpreter
Term neonates	• From birth up to the maximum 72 hours post-birth • Gestational age ≥37 weeks • Weight ≥ 2500 grams • APGAR Score ≥7 at five minutes	
Paediatric	• Attending hospital for minor elective surgery requiring general anaesthetic (30 days to 18 years) OR volunteer to participate in the study after seeing a flyer related to the study (15 to 18 years)	
Category		
Haematology		• Presence of coagulation disorders • Family history of coagulation disorders • Currently on anticoagulation medication
Immunology		• Presence of immune system disorder or immune deficiency syndrome • Presence of genetic disorder

		• Presence of rheumatologic disorder • Family history of rheumatologic disorder or immune deficiency syndrome • Infection or a febrile illness within the last 7 days • Infection or a febrile illness within the las 4 weeks • Hospital admission for intravenous (IV) antibiotics to clear an infection on more than 2 occasions in life • Has needed 2 or more months of oral antibiotics more than 2 occasions in their life • Failure to thrive • Recipient of blood products in the last 3 months • Diagnosed with food allergy, asthma, eczema or hayfever • Family history of food allergy, asthma, eczema or food allergy
Biochemistry		• History of liver and renal disease • Presence of endocrine diseases • Presence of metabolic disease • Presence of renal disease • Presence of hepatic disease • Failure to thrive

Sample size

A minimum of 20 samples is collected for 19 specific age groups e.g. neonates and every year of life from 30 days to 18 years [1]. For the biochemical and haematological parameters, a total of 380 samples are analysed. For the immunological analysis, the number of age groups is increased from 19 to 22 (i.e. 1 to 2 months, 2 to 6 months, 6 to 9 months, 9 to 12 months, 12 to 18 months, 18 to 24 months, and every year of life from 3 years to 18 years), equating to a total of 440 samples. For pre-term neonates, a total of 100 samples are collected to facilitate analysis of 20 samples for each week of gestation from 32 to 36 weeks.

The sample size of 380 or 440 for estimating 95% reference intervals of an analyte using parametric methods with samples distributed uniformly across 30 days to < 18 years is considered adequate based on the approach proposed by Royston [1]. Using a formula for the approximate standard error of the reference limit at the mean value of age, he suggested 292 as a suitable sample size for

constructing a 95% reference intervals based on restricting the standard error of the limits of the resulting reference intervals to be no more than 10 percent of the residual standard deviation from the parametric model [1].

Sample processing and storage

Samples are thawed as per standard clinical laboratory practice for the analyte in question. There are no repeat freeze-thaw cycles for any of the samples. For some analytes the samples are processed immediately and not frozen, within the same time frame and conditions under which clinical samples would be handled. Analytes that can be frozen will be tested in batches. Testing will be done with samples from all ages included in each batch with randomly assigned orders of testing to avoid any batch to batch bias.

For biochemistry analytes, samples are collected into S-Monovette serum gel tubes (Sarstedt), centrifuged at 5000rpm and 6 °C for 5 minutes, separated and stored in 400 µL aliquots at -80 °C within four hours of collection.

For immunology analytes, samples are collected into either S-Monovette serum gel tubes (Sarstedt), or S-Monovette neutral tubes (Sarstedt), depending on the downstream tests. The samples are spun at 5000rpm and 6 °C for 5 minutes, separated and the resultant serum stored in 400µL aliquots at -80 °C within four hours of collection.

For haematology analytes, samples are collected into S-Monovette citrate 3.2% tubes (Sarstedt). The samples are centrifuged at 3800rpm at room temperature for 5 minutes, and plasma is stored in 500µL aliquots at -80 °C within four hours of collection, using a previously established collection protocol [13].

Samples utilised for determination of blood groups (ABO) and the thalassemia screen are collected into 500uL S-Monovette EDTA tubes and processed in the laboratory within 24 hours of collection.

Cell count samples are collected in S-Monovette EDTA tubes (Sarstedt) and processed by the laboratory within 3 hours of collection.

The details of blood tubes used in sample collection is provided in table 2.

Table 2: Blood tubes used in sample collection

Analytes catogy	Tube Type	Manufacturer	Subtype	Volume	Product Code	Age group

Biochemistry	SST	Sarstedt	S-Monovette	7.5 mL	01.1602.001	Neonate and Paediatric
Haematology	EDTA	Sarstedt	S-Monovette	2.7 mL	05.1167.001	Paediatric
	EDTA	Sarstedt	Micro Tube	0.5 mL	41.1395.002	Neonate
	Sodium Citrate	Sarstedt	S-Monovette	3 mL	05.1165.100	Paediatric
	Sodium Citrate	Sarstedt	S-Monovette	1.4 mL	06.1668.100	Neonate
	Lithium Heparin	Sarstedt	S-Monovette	7.5 mL	01.1608.001	Paediatric
	Lithium Heparin	Sarstedt		0.5 mL	20.1345	Neonate
Immunology	SST	Sarstedt	S-Monovette	7.5 mL	01.1602.001	Neonate and Paediatric
	Neutral	Sarstedt	S-Monovette	7.5 mL	01.1728.001	Neonate and Paediatric

Sample testing

Blood samples collected from the study participants are tested according to documented and accredited standard operating procedures used for the testing of patient samples within the participating laboratories. The biochemical, immunological and haematological analytes tested are listed in table 3. Where indicated (*), sample aliquots from the same patient are tested for the same analyte using different automated analysers commonly in clinical use to facilitate direct head to head comparison. In addition, where indicated in table 3 (†), aliquots of the same sample are tested in different laboratories using the same analyser type and test method. The sample testing is currently ongoing.

Table 3: List of analytes tested for the HAPPI Kids study.

Biochemical	Immunological	Haematological
Sodium*††	Immunoglobulin G*	Factors: XII, XI, IX, X, II, VII, VIII, V
Potassium*††	Immunoglobulin A*	Inhibitors: Protein C, Protein S, Antithrombin, Alpha-2-macroglobulin
Chloride*††	Immunoglobulin M*	Von Willebrand factor
Bicarbonate*††	Rheumatoid factor*	Collegen binding assay

Urea*+†	Complement component 3*	Ristocetin cofactor assay
Creatinine *+†	Complement component 4*	Activated Partial Thromboplastin Time†
Total Bilirubin†	Cystatin C*	Prothrombin Time†
Conjugated Bilirubin*+†	ANTI-STREPTOLYSIN O TEST*	Fibrinogen†
Alkaline Phosphate*+†	Thyroid peroxidase*	Thrombin clotting time
Aspartate Aminotransferase *+†	Thyroglobulin*	D-Dimers†
Alanine Aminotransferase *+†	Iron*	Full Blood Examination and Reticulocytes
Gamma-Glutamyl Transferase *+†	Ferritin*†	Red Cell Folate
Total Protein*+	Transferrin*	Glucose-6-phosphate dehydrogenase
Albumin*+	Antinuclear antibody	Active B12
Calcium*+†	Soluble FAS Ligand	Total Homocysteine
Magnesium*+†	Soluble CD25	Total B12
Phosphate*+†	Immunoglobulin E	Serum Folate
Lactate dehydrogenase*+	Classic Haemolytic Complement Pathway	
Creatine Kinase *+	Alternative Haemolytic Complement Pathway	
Lipase*+	Mannose-binding lectin Complement Pathway	
Amylase*+		
Uric Acid*+		
Triglycerides *+		
Cholesterol*+		
High-density lipoprotein*+		
Thyroid Stimulating Hormone *+		
Free Thyroxine *+		
Free Triiodothyronine *+		
Anti-Müllerian hormone *		
Oestradiol*		
Sex hormone binding globulin *		
Dehydroepiandrosterone – Sulphate*		
Cortisol*		
Growth Hormone*		
Testosterone*		
25-hydroxyvitamin D*		
High Sensitivity Oestradiol*		
Androstenedione*		
17α-Hydroxyprogesterone *		
Insulin-like growth factor 1*		
Insulin-like growth factor-binding protein-3 *		

* Sample aliquots from the same patient are tested for the same analyte using different automated analysers in common clinical use to facilitate direct head to head comparison.

+ Aliquots of the same sample are tested in different laboratories using the same analyser type and test method.

† Samples from pre term children are tested.

Data management system

Study data are collected and managed using the REDCap (Research Electronic Data Capture) electronic data capture system hosted at the Murdoch Children's Research Institute [14]. REDCap is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

Data analysis

Test results for different analytes will be plotted according to age in order to identify outliers, explore normality of distributions, and to assess the association between analyte level and age. Outliers will to be excluded from analysis following a detailed examination, including visual inspection, appropriate statistical tests e.g. Tukey's test and assessment of biological implausibility e.g. haemolysis index for biochemistry samples. Reference intervals will be constructed using parametric statistical methods in two steps. In the first step, a fractional polynomial regression model of age will be fitted to the mean of normally distributed or log transformed test results of an analyte, using sex as a covariate [15]. In the second step, the 2.5^{th} and 97.5^{th} centiles will be estimated using quantile regression where the power variables of age from the fractional polynomial regression model are used as covariates [16]. Potential interaction with sex will be examined. The 95% confidence interval of the reference intervals will be estimated based on the fitted model for the reference limits. A combination of statistical testing, i.e. goodness of fit and variance component analysis, and clinical expertise will be used to determine the extent to which the reference intervals of an analyte based on different analysers can be compared.

Stata 15 will be used for data analysis [17].

Study timeframe

The participant recruitment and sample collection commenced on February 2015 and is expected to be completed by August 2021. The testing of samples for 30 biochemical analytes has commenced, while testing of samples for the remaining biochemical analytes and immunological and haematological analytes are currently on going. We plan to complete the testing of sample by February 2021.

Patient and public involvement

The study was conceived in response to patient complaints about having blood tests done at community pathology laboratories and their children requiring repeat testing because age-appropriate normal data was not available. The recruitment and consent processes were piloted and parental/ child feedback incorporated into the main study protocol. A summary of the results will be shared by mail with all the participants involved. If any child has a clinically abnormal result the parents are contacted and appropriate plans for review of the child are made.

ETHICS AND DISSEMINATION

The study protocol has been approved (34183 A) by The Royal Children's Hospital, Melbourne, Ethics in Human Research Committee (HREC) and subsequently approved by the HREC committees of all participating hospitals including, The Royal Women's Hospital, Northern Health and Western Health - Sunshine Hospital. The study is primarily supported by The Royal Children's Hospital Foundation and is based at the Haematology Research Laboratory, Murdoch Children's Research Institute, Melbourne, Australia.

Written consent is obtained from parents or guardians of eligible study participants following a verbal and written explanation of the study and participation requirements by Pathology Collection staff at each site after. Less than 3% of the blood volume is collected from a child considering the amount of blood volume a child has, while only one attempt is made to minimise risk associated with collecting intravenous blood.

Participants' identifying information is replaced by a study number (ID) at recruitment to maintain confidentiality. This ID is used in all laboratory specimens, evaluation forms, reports and other records that leaves the site. Electronic data are stored in REDCap which can only be accessed by the authorised members of the research team. Similarly, paper-based information are stored in a locked filing cabinet and can only be accessed by the designated persons. The blood samples are stored in freezers that are only accessible to authorised members of the research team by using their individual swipe card maintaining a record of who has accessed the samples and at what time.

All results that fall outside the existing reference range used by the clinical laboratory are referred immediately to the study coordinator to determine whether there is any clinical significance that requires follow-up for the child. If necessary, families are contacted and the blood tests repeated, or an appropriate referral will be made and the individual will be followed up by the appropriate specialist.

At the conclusion of the study, blood samples collected as part of this study will be destroyed, unless consent has been given for them to be stored in a biobank located at the Haematology Research Laboratory for use in future research.

The study results will be summarised for submission to peer-reviewed journals. Results will be shared with participating hospitals and laboratories and presented at local meetings, national and international conferences.

DISCUSSION

Reference intervals are used on a daily basis by clinicians to interpret measurements obtained from patients [18]. The accuracy and reliability of the reference intervals are highly dependent on the reference population utilised to define those reference intervals [11, 19]. As recommended by Harris and Boyd[], the characteristics of the sample from the reference population should be similar to those of the patients [20]. Paediatric reference intervals established by laboratories using retrospective data mining techniques provide extensive sample numbers. However, the representativeness of the population is often compromised by using the laboratory results of children who were initially referred for a significant clinical investigation [8]. Samples collected prospectively from pre-schools are useful in constructing reference intervals, but are applicable to pre-school aged children only [6]. Addressing this issue, the Canadian Initiative in Paediatric Reference Intervals (CALIPER) and German Health Interview and Examination Survey for Children and Diagnosis (KiGGS) collected data prospectively using a community-based approach ensuring representativeness [10, 21, 22]. Similarly, the HAPPI Kids study is one of the very few studies collecting data prospectively from pre-defined healthy preterm neonates and term neonates from birth until 72 hours post birth, as well as children from 30 days to 18 years of age.

Samples collected for HAPPI Kids utilise paediatric Pathology Collectors with specific skills and documented competency to collect venous samples from neonates. All collected samples meet RCH laboratory standard operating procedures for collection and storage as documented in the Specimen Collection Handbook (SCH) for the analytes [23]. All participating laboratories perform daily quality control, participated in external quality assurance programs (Royal College of Pathologists of Australasia Quality Assurance Programs (RCPA QAP)) and were accredited by the National Association of Testing Authorities (NATA). The prospective design of the HAPPI Kids study also ensures quality of sample collection, storage and testing in different laboratories.

The CLSI recommended sample size for establishing reference intervals for a discrete age group is 120 [12]. However, there is no recommendation for sample size when reference intervals are

constructed for continuous age. Few papers have previously reported continuous reference intervals across childhood and are based on retrospective data mining of samples taken for clinical purposes over many years [4, 24]. No previous studies have collected prospective samples (which were not required for clinical management) from neonates and children specifically screened to be a "well population" for the purpose of developing continuous reference intervals. In that context the exact numbers required remain unknown. We judged that a similar degree of precision in estimation of the limits of agreement to that proposed by Royston (1991), i.e. requiring the ratio of the SE of the estimated limits to be no greater than 10% of the SD of the variation in the population, would be appropriate in our setting. Planning to recruit 380 children assured us of meeting this criterion. [1]. However, it is our intention to do a simulation study to develop better guidance for appropriate numbers of samples required to allow reliable characterisation of age-specific analyte dynamics.

The direct comparison of reference intervals by analysers is not possible using data mining techniques, nor when testing remainder of clinically driven samples (due to sample aliquot limitations). To overcome this problem CALIPER program which initially established reference intervals using the Abbott ARCHITECT analyser transferred reference intervals in line with Clinical & Laboratory Standards Institute (CLSI) recommendations by testing only 100 samples on Beckman Coulter DxC800, Ortho Vitros 5600, Roche Cobas 600 and Siemens Vista 1500 using an algorithm [11, 12]. The study used $r^2 < 0.70$ as an indicator of non-transference between Abbott ARCHITECT and another platform and reported several analytes as non-transferable between analysers[11]. The challenges in transference from the Abbott ARCHITECT to another analyser were discussed by the authors including the lack of an appropriate number of samples per age group since only 100 samples were tested to predict the linear relationship [11]. The HAPPI Kids study utilises aliquots of the same blood sample from the same patient and tests the aliquots on different analysers, enabling the first documented head-to-head comparison between analysers for the age spectrum covering neonates to children 18 years of age. Therefore, we expect the results of the HAPPI Kids study to be very useful in improving interpretation of pathology results for neonates and children.

REFERENCE

1. Royston, P., *Constructing time-specific reference ranges.* Statistics in Medicine, 1991. **10**(5): p. 675-690.
2. Coffin, C.M., et al., *Pediatric laboratory medicine: current challenges and future opportunities.* American Journal Of Clinical Pathology, 2002. **117**(5): p. 683-690.
3. Adeli, K., *Closing the gaps in pediatric reference intervals: The CALIPER initiative.* Clinical Biochemistry, 2011. **44**(7): p. 480-482.
4. Loh, T.P., et al., *Development of paediatric biochemistry centile charts as a complement to laboratory reference intervals.* Pathology, 2014. **46**(4): p. 336-43.

5. *Pediatric reference intervals: Critical gap analysis and establishment of a national initiative.* Clinical Biochemistry, 2006. **39**(6): p. 559-560.
6. Southcott, E.K., et al., *Establishment of pediatric reference intervals on a large cohort of healthy children.* Clinica Chimica Acta, 2010. **411**(19–20): p. 1421-1427.
7. Shaw, J.L.V., et al., *Validity of establishing pediatric reference intervals based on hospital patient data: A comparison of the modified Hoffmann approach to CALIPER reference intervals obtained in healthy children.* Clinical Biochemistry, 2014. **47**(3): p. 166-172.
8. Colantonio, D.A., et al., *Closing the gaps in pediatric laboratory reference intervals: a CALIPER database of 40 biochemical markers in a healthy and multiethnic population of children.* Clin Chem, 2012. **58**(5): p. 854-68.
9. Daly, C.H., et al., *A systematic review of statistical methods used in constructing pediatric reference intervals.* Clinical Biochemistry, 2013. **46**(13–14): p. 1220-1227.
10. Kohse, K.P., *KiGGS — The German survey on children's health as data base for reference intervals and beyond.* Clinical Biochemistry, 2014. **47**(9): p. 742-743.
11. Estey, M.P., et al., *CLSI-based transference of the CALIPER database of pediatric reference intervals from Abbott to Beckman, Ortho, Roche and Siemens Clinical Chemistry Assays: Direct validation using reference samples from the CALIPER cohort.* Clinical Biochemistry, 2013. **46**(13–14): p. 1197-1219.
12. CLSI, *CLSI Releases Guidelines for Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory*. 2008, COMTEX News Network, Inc.
13. Monagle, P., et al., *Developmental haemostasis. Impact for clinical haemostasis laboratories.* Thromb Haemost, 2006. **95**(2): p. 362-72.
14. Harris, P.A., et al., *Research Electronic Data Capture (REDCap) - A metadata-driven methodology and workflow process for providing translational research informatics support.* Journal of biomedical informatics, 2009. **42**(2): p. 377-381.
15. Royston, P. and E.M. Wright, *A method for estimating age-specific reference intervals ('normal ranges') based on fractional polynomials and exponential transformation.* Journal of the Royal Statistical Society: Series A (Statistics in Society), 1998. **161**(1): p. 79-101.
16. Wei, Y., et al., *Quantile regression methods for reference growth charts.* Statistics in Medicine, 2006. **25**(8): p. 1369-1382.
17. StataCrop. *Stata Statisical Software: Release 15.* 2017. Colleage Station, TX: Stata Crop LP.
18. Koerbin, G., J.R. Tate, and A.C.f.C.R.I. on behalf of the, *Harmonising Adult Reference Intervals in Australia and New Zealand – the Continuing Story.* The Clinical Biochemist Reviews, 2016. **37**(3): p. 121-129.
19. Ritchie, R.F. and G. Palomaki, *Selecting clinically relevant populations for reference intervals.* Clinical Chemistry And Laboratory Medicine, 2004. **42**(7): p. 702-709.
20. Eugene K. H and James C. B,*Statistical Bases of Reference Values in Laboratory Medicine,. New York; Marcel Dekker, Inc., 1995.*
21. Adeli, K., et al., *The Canadian laboratory initiative on pediatric reference intervals: A CALIPER white paper.* Crit Rev Clin Lab Sci, 2017. **54**(6): p. 358-413.
22. Ridefelt, P., *Pediatric reference intervals - the Swedish experience.* Clin Biochem, 2014. **47**(9): p. 740-1.
23. Melbourne, T.R.C.s.H. *RCH Specimen collection handbook*. Available from: https://ww2.rch.org.au/specimen/.
24. Zierk, J., et al., *Age- and Sex-Specific Dynamics in 22 Hematologic and Biochemical Analytes from Birth to Adolescence.* Clin Chem, 2015. **61**(7): p. 964-973.

AUTHOR STATEMENT

MH wrote the manuscript, and developed the statistical analysis plan. VK wrote the study protocol, obtained ethics approval for the study, co-wrote the manuscript and is the co-ordinator of the study. SM provided Biochemistry expertise in the design of the study and revised the manuscript. JB provided pathology collection expertise in the design of the study, training for the pathology collectors and reviewed the manuscript. JC and SD provided support for statistical analysis plan and reviewed the manuscript. PM conceived the study and contributed to its design and was a major contributor in writing the manuscript. VI contributed to the design of the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

FUNDING

This study has been primarily funded by The Royal Children's Hospital Foundation (Proposal ID: 2014 – 233), with supplementary funding from Ortho Diagnostics. In addition, the study received in-kind reagents from Roche Diagnostics. Funding bodies do not have any input in the design of the study, sample collection, analysis and interpretation of data and in preparation of the manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

The authors are grateful to the parents and children for their participation in this study. The authors thank members of the Pathology Collection Department of The Royal Children's Hospital for the consent of participants and collection of samples. The authors acknowledge the contribution of the members of the anaesthetic and surgical departments of the Royal Children's Hospital, Northern Hospital and Western Health - Sunshine Hospital, Melbourne, for facilitation of sample collection. In addition, the authors thank members of the post-natal departments of The Royal Women's Hospital, Northern Hospital and Western Health, Sunshine Hospital.

Supplementary table 1: Questionnaire for assessing inclusion and exclusion criteria.

Participant/Category	Questions / Data fields
All Sample	Study ID
	Date of Recruitment
	Site of Recruitment
	Date Sample Collected
	Hospital
	UR
	First Name
	Surname
	Date of Birth
	Sex
	Address
	Ethnicity of Mother
	Ethnicity of Father
	Tube Type Collected and Volume of blood collected
Neonates	Gestation
	Time of Birth
	Day of Bleed
	Nature of Delivery
	Weight
	APGAR Score at 5 minutes
	Feeding Method
	Date/Time of Vitamin K
	Current Medications
Paediatric	Procedure performed
	Current Medications
	Clinical Information
Haematology	Dose the participant have a presence of coagulation disorders?
	Does the participant have a family history of coagulation disorders?
	Is there participant currently on anticoagulation medication?
Immunology	Does the participant have a presence of an immune system disorder or immune deficiency syndrome?
	Does the participant have a presence of a genetic disorder?
	Does the participant have a presence of a rheumatologic disorder?
	Has any family member or relative of your child ever been diagnosed with an immune system disorder or immune deficiency syndrome?
	Has the participant had an infection or a febrile illness within the last 7 days?

	Has the participant had a hospital admission for intravenous (IV) antibiotics to clear an infection on more than 2 occasions in life?
	Has the participant needed 2 or more months of oral antibiotics more than 2 occasions in their life
	Does the participant have a history of a failure to thrive?
	Has the participant been a recipient of blood products in the last 3 months?
	Has a doctor ever diagnosed your child with food allergy, asthma, eczema or hayfever?
	Has anyone in your child's immediate family (that is, your child's parents and/or siblings) ever been diagnosed with food allergy, asthma, eczema or hayfever?
Biochemistry	Does the participant have a history of liver and renal disease?
	Does the participant have a presence of endocrine diseases?
	Does the participant have a presence of metabolic disease?
	Does the participant have a presence of renal disease?
	Does the participant have a presence of hepatic disease?
	Does the participant have a history of failure to thrive?