Appendix J. Technical Stable Isotope Methods

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Introduction

Two protocols were followed for the stable isotope component of the clinic visit. Protocol 1 (breastfed protocol) provided a measure of breast milk intake, and Protocol 2 (non-breastfed protocol) was intended to provide body composition and fluid intake.

This appendix describes in detail the modelling of the data from both protocols, the technical stable isotope methods and the results for water turnover and water flux. Results for the volume of breast milk consumed are presented in chapter 5. Results for body composition and fluid intake are not presented in this report, due to the difficulties experienced in accurately estimating stable isotope losses.

J.1. Models used in data calculation

The data was modelled using either the breast milk intake model as described in section J.1.1 or using the water turnover model as described in section J.1.2.

J.1.1. Protocol 1 – Breast Milk intake model

Breast milk volumes were calculated using the dose-to-mother[1] technique as described in Haisma et al[2].

The dose-to-mother method is an accurate method of determining the amount of breast milk received by a breastfed child. The principle of the method is that a tracer dose (deuterium rich) is administered to the mother, and then by using the rate of deuterium appearance and disappearance in the child, it is possible to calculate the amount of breast milk consumed.

The tracer water is incorporated into the mother's body water pool and is passed onto her baby as breast milk. Below is a diagram showing the model, where water flow is shown by the arrows.

Figure J.1. Model of water and tracer flow in the dose-to-mother method.
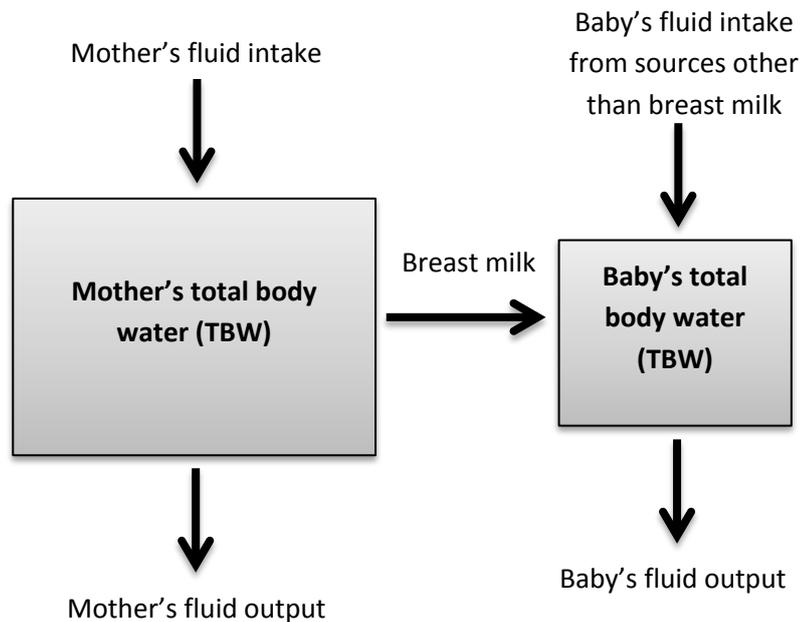
Mathematical analysis of the model indicates that the curve of the mother's body water enrichment should follow an exponential decay, whereas the child's enrichment curve is more complicated, with the curve starting from zero, reaching a maximum and then falling.

To a first approximation, for an exclusively breastfed child, when the mother's and child's enrichment curves are plotted on the same graph, as shown in Figure J.2, the mother's curve crosses that of the child at its maximum value.

For a child who is fed complementary foods alongside breast milk, the maximum of the child's curve falls below the mother's curve, as shown in Figure J.3. The relative heights of the child's and mother's curves at this time represent the fraction of feeding due to breast milk.

If the child is known not to be breastfed at all, there is clearly no point in giving the mother a tracer dose as this will not be passed onto the child via her breast milk.

The model works by exploiting the established fact that body water can be represented by a single pool for measurements made over the timescales of such studies (typically two weeks). The model adopted consists of two compartments (one representing the mother and the other the child) with unidirectional flow between them. Since the experimental measurements are of tracer concentration, water flux can only be fully determined if either the dose given or the water pool size is known. For the mother the size of the dose administered is measured precisely, but for the child neither the amount of

incoming tracer, nor the size of the body water pool into which it is diluted is known. For this reason an independent assessment of the child's body water was made, obtained from a predictive equation. An independent assessment using a second tracer was attempted; however in many cases the quantity of the dose consumed by the child was not measured with sufficient accuracy, and so a predictive equation was used. The prediction equation used is the one quoted in Haisma et al[2].

Once the kinetics of water flow, corrected for isotope fractionation, have been determined the amount of breast milk consumed by the child is obtained using assumptions about the water content of breast milk, and also the contribution to the child's water pool due to the oxidation of milk solids. The rate of loss of tracer from the baby is used to determine the total efflux of water, from which the amount of breast milk derived water is subtracted together with an estimate of water lost in breath, to give an estimate of oral water intake due to sources other than breast milk.

Figure J.2. Kinetics of isotope enrichment of maternal and infant body water for an exclusively breastfed child
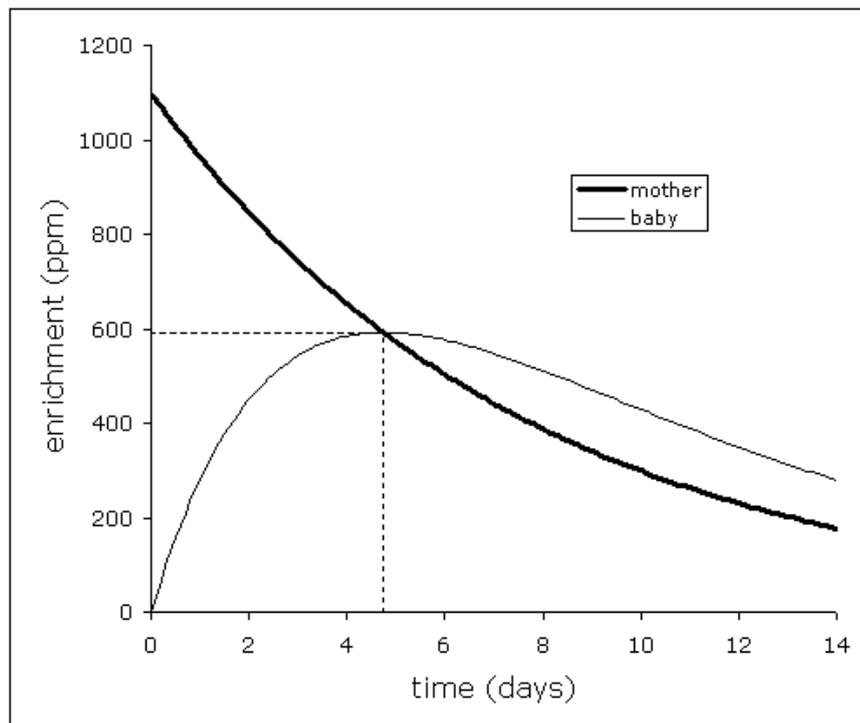
Figure J.3. Kinetics of isotope enrichment of maternal and infant body water for a child fed breast milk and complementary foods
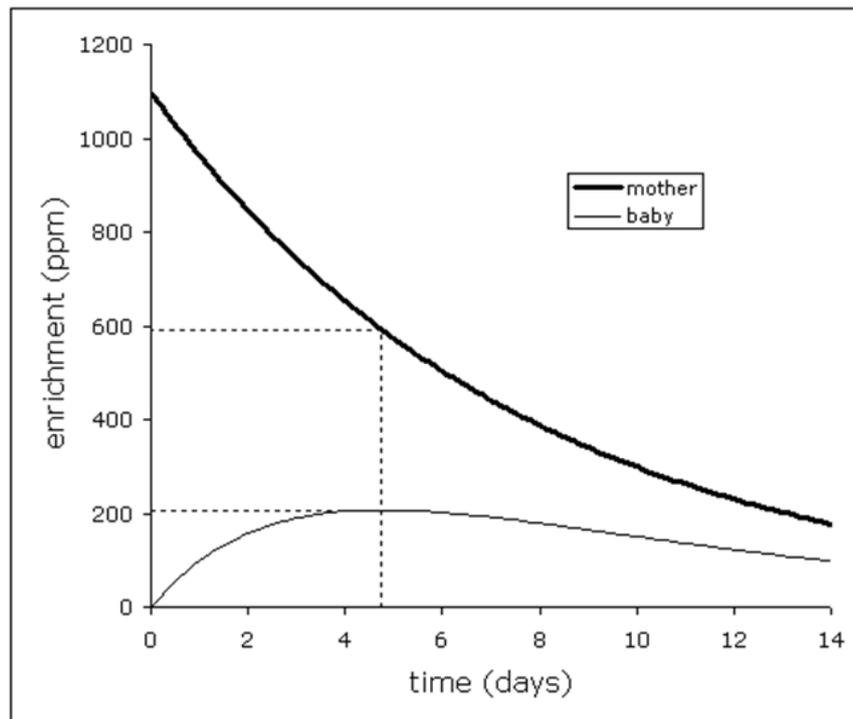
J.1.2. Protocol 2 – Water Turnover

For children not taking part in Protocol 1, measurements of water turnover and water flux were still desirable. These were obtained by administering a dose of deuterium oxide to the child, and collecting urine for five days.

The water turnover model is far simpler than the breast milk intake model. Here, the disappearance of the tracer from a single pool is measured. As in the mother's case for Protocol 1, tracer is lost with an exponential rate of decay. By plotting the natural logarithms of the parts per million excess and fitting a line through the data, the slope can be calculated. The slope provides the fractional rate of daily water turnover. Therefore, a slope of -0.20 would imply that the daily water turnover is 0.2 of the whole water pool, or alternatively 20% of the water pool is lost and replaced each day. Using the same predictive equation for body water as for the Protocol 1 children, allows for calculation of the water flux from the child.

J.1.3. Percentage water turnover and water flux

The following variables were derived for both protocols:

- Percentage water turnover (% per day) is the percentage of the body water pool that is replaced each day.
- Water flux (L per day) is the amount of the body water pool in litres that is replaced each day.
- Water flux (L per kg per day) is the water flux (L per day) adjusted by the body weight.

For participants taking part in the breastfed protocol, the water flux (L per day) was calculated; this enabled calculation of the percentage water turnover as the relationship between the two variables is as follows:

$$Water\ flux = k \times TBW \qquad (Equation\ 1)$$

Where water flux is expressed in litres per day, k represents the water turnover expressed as a fraction of the body's water pool in units per day, and TBW is an estimate of total body water (the body's water pool) in litres.

TBW is estimated using the equation as described in Haisma et al (2003)[2], given below:

$$TBW = 0.84 \times WT^{0.82} \qquad (Equation\ 2)$$

Where WT is the child's weight in kilograms.

Water flux may also be expressed as:

$$Water\ flux\ (L\ kg^{-1}day^{-1}) = \frac{Water\ flux\ (L\ day^{-1})}{WT(kg)} \qquad (Equation\ 3)$$

For participants taking part in the non-breastfed protocol, the water turnover is directly calculated and this is then used to derive water flux, as in equation 1. TBW is calculated according to equation 2.

J.2. Hydrogen Isotope Analysis

The abundance of deuterium (^{2}H) in the urine samples was determined by gas isotope ratio mass spectrometry (GIRMS).

A 200µL aliquot of urine was placed in a 3.7ml vial along with a platinum catalyst (platinum metal mounted on a hydrophobic polymer substrate). The vial was then flush filled with hydrogen gas at approximately 200mBar pressure and left to equilibrate at 22±0.1°C for at least six hours.

Equilibration took place by exchange of the hydrogen in the hydrogen gas (^{1}H-^{1}H) with the hydrogen in the water (^{1}H-^{2}H-O), which was facilitated by the platinum catalyst.

$$^1H\text{-}^2H\text{-}O + {}^1H\text{-}^1H \rightarrow {}^1H\text{-}^1H\text{-}O + {}^1H\text{-}^2H$$
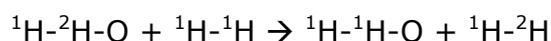
This resulted in the headspace gas having an isotopic composition of ^{2}H and ^{1}H that reflects the isotopic composition of the sample.

Once equilibration was achieved, the isotopic composition of the headspace gas in each vial was determined using a dual inlet isotope ratio mass spectrometer (either a Sira 10, V.G Instruments, Middlewich, Cheshire; or an Isoprime, G.V.Instuments Ltd, Wythenshawe, U.K.). The gas was dried cryogenically before being admitted to the sample bellows of the dual inlet instrument. The volumes of the sample and reference bellows were then adjusted by the instrument so that the same partial pressure of hydrogen gas is produced when connected to the mass spectrometer ion source.

The inlet was rapidly switched between the sample gas (^{1}H-^{2}H or mass 3) and reference gas (^{1}H-^{1}H or mass 2) and a number of measurements were made. The ratio of the ion beam intensities at mass 3 and mass 2 were recorded. N.B: A hydrogen atom ^{1}H has a mass of 1, so hydrogen gas ^{1}H-^{1}H has a mass of 2. Deuterium (^{2}H) is one mass unit heavier than ^{1}H, so hydrogen gas ^{1}H-^{2}H has a mass of 3.

Before further processing, these ratios were corrected for the contribution of H_3^+ to the mass 3 beam, due to chemical ionisation (protonation) of $^1H_2$.

$$^1H^+ + {}^1H\text{-}^1H \rightarrow {}^1H_3^+$$
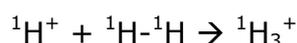
The ion beam measurements and calculations to this point were made by the manufacturers' software, and reported as a delta value for the sample with respect to the working reference (WR) i.e. an arbitrary sample of hydrogen gas. This delta value was defined as:

$$\delta_{sample,WR} = 1000 \times \left(\frac{R_{sample}}{R_{WR}} - 1 \right)$$

Samples of specially prepared water standards were included in each batch of samples run on the instrument. These standards have isotopic compositions in the range of those anticipated in the samples and can be traced back to an internationally defined primary standard, called Vienna Standard Mean Ocean Water (V-SMOW). Using the above water standards, a two-point calibration was done, the so-called SMOW/SLAP correction (SLAP here refers to Standard Light Antarctic Precipitation – another international primary standard).

Expressions were obtained describing the isotopic composition analogous to the delta value described earlier, but now referenced to V-SMOW instead of the working standard, as shown below.

$$\delta_{sample,VSMOW} = 1000 \times \left(\frac{R_{sample}}{R_{VSMOW}} - 1 \right)$$

V-SMOW is known to have an isotopic composition, R_{VSMOW}, of 155.76 ±0.1 ppm (parts per million). By rearranging the above equation, the isotopic composition of R_{sample} in parts per million can be obtained.

J.3. Oxygen Isotope Analysis

The abundance of oxygen-18 (^{18}O) in the urine samples was also determined by gas isotope ratio mass spectrometry (GIRMS).

Aliquots of 250µl of urine were placed in 12ml vacutainers (Labco Ltd, High Wycombe, UK) and flush filled with 5% CO_2 in nitrogen. Samples were then equilibrated overnight at room temperature before analysis. Measurements of $^{18}O/^{16}O$ ratios were made using an AP2003 continuous flow IRMS (Analytical Precision Ltd, Northwich, Cheshire, UK).

Delta values of isotopic composition of the samples were obtained and expressed relative to V-SMOW as described in J.1.

J.4. Quality Control

A number of quality control measures were applied both during the analyses of samples and also when calculating parameters using the above models.

J.4.1. Analysis Quality Control

Standards were run at the beginning, middle and end of every run and a correction applied to monitor any drift in repeated measurements. Any drift of more than ten deltas difference between the beginning, middle and end of the run was being monitored and samples were repeated for this reason.

In addition, standards were randomly placed amongst the 'unknown' samples in each run. These have enrichments similar to what is expected in the samples. Differences between repeated measurements of these random standards are monitored for any variations. If there was a variation of more than 1-2% then next run's standards were monitored or samples repeated. Differences can also be seen if a fault is found on the instrument or a part is replaced or the tunings

have changed. As a check, a run of our internal water standards of known enrichments was run before sample analysis could resume.

In addition to each participant's samples, a sample of the tracer water dose drunk was analysed. In large-scale studies, batches of tracer water doses are being prepared on an almost daily basis. This means that a number of participants would have received a tracer water dose from the same batch of dose prepared on one day. During analysis, a sample of this batch of dose was firstly diluted and then run with each participant's samples. Monitoring the enrichment of this dose was another quality control check, since the enrichment of a dose from the same batch should be almost identical. Small deviations in enrichment of the dose were monitored and repeated if required; any large deviations could indicate a possible contamination of the dose.

J.4.2. Data Quality Checks:

Additional result checks included looking at the actual delta values of individual samples and the expected fate of the tracer in the participant's body i.e. the shape of the curve for Protocol 1 or the slope of the line of best fit for Protocol 2. Any data points not following these were repeated to ensure duplicate measures and exclude any machine errors. The date and time of sample collection and any other parameters such as the amount of dose drunk, were also checked to ensure all parameters needed for the model calculations were correct.

J.5. Results for water turnover and water flux

Water turnover and water flux results for both protocols are presented in Addendum 1 (Tables J.1 and J.2). No statistical testing was carried out and therefore any noted differences are only observations.

For both protocols, there was an overall trend for the percentage water turnover per day to decrease with age. As a child grows, the water pool becomes larger and the proportion of it that is replaced each day becomes smaller. However, only a minimal change in percentage water turnover per day was observed with age in the non-breastfed protocol participants over 10 months old (Figure J.A).

For both protocols, no changes were observed for weight-adjusted water flux (L per kg body weight per day).

Water flux (L per day) was greater in the non-breastfed children than in the breastfed children. This is expected as the children in the non-breastfed protocol were heavier than their breastfed counterparts.

Also, for the non-breastfed children an increase in water flux (L per day) across age groups was observed. As the child grows, fluid intake is greater and hence the water flux (L per day) increases (Figure J.B).

**Figure J.4.** Mean water turnover (%) with age for breastfed and non-breastfed protocols
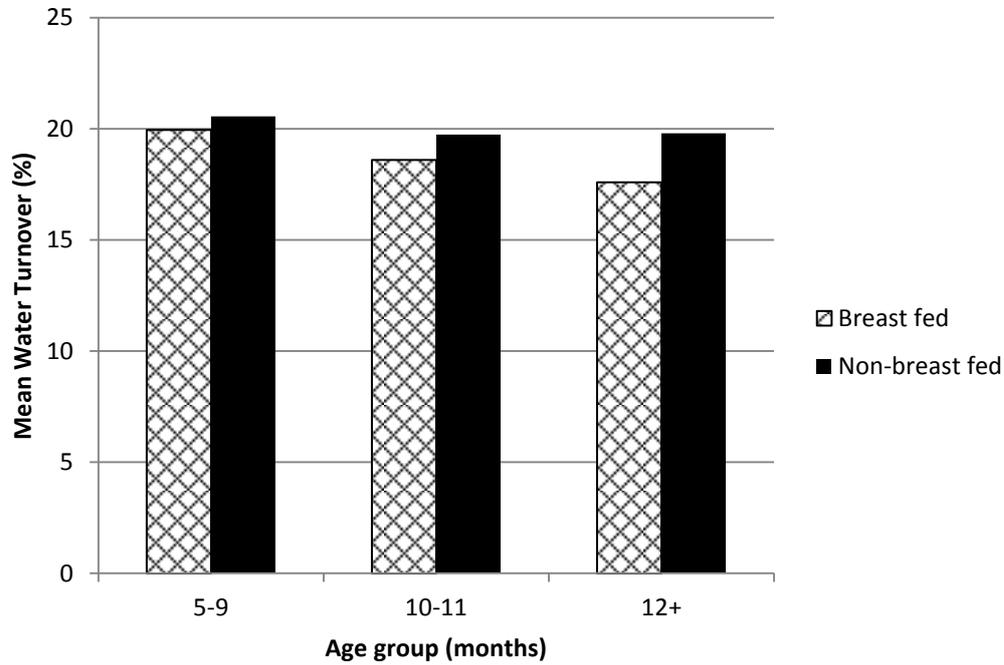
Figure J.5. Mean water flux (L/day) with age for breastfed and non-breastfed protocols

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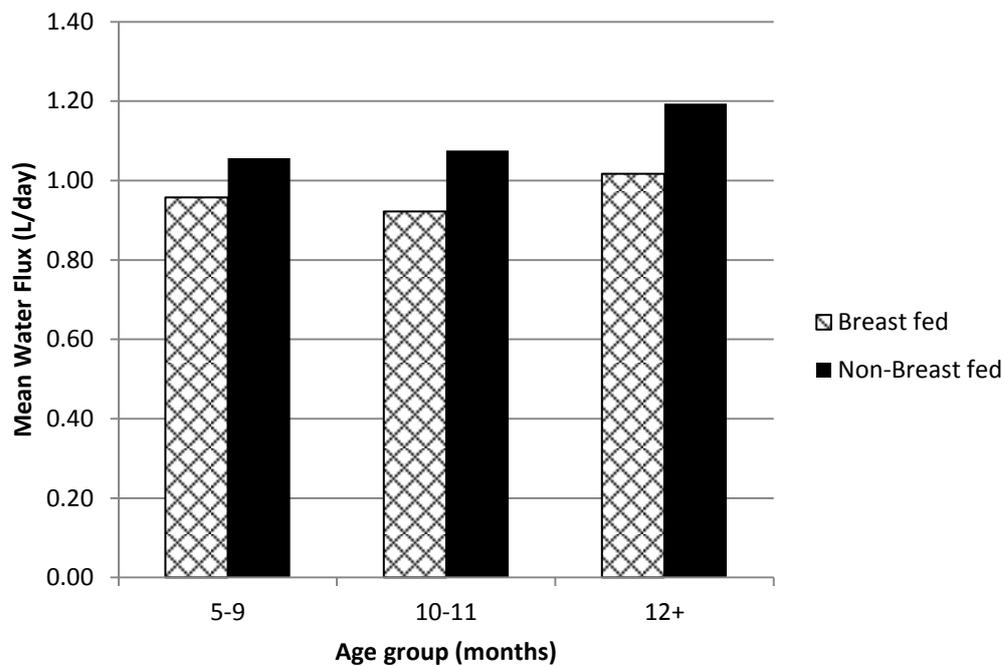
References and endnotes

[1] http://www-pub.iaea.org/MTCD/publications/PubDetails.asp?pubId=8168

[2] Haisma, H., W. A. Coward, E. Albernaz, G. H. Visser, J. C. K. Wells, A. Wright and C. G. Victoria (2003). Breast milk and energy intake in exclusively, predominantly, and partially breast-fed infants. *European Journal of Clinical Nutrition* 57, (12), 1633-1642.

Addendum

Table J.1
Percentage water turnover per day and Water flux measured by stable isotopes

	Breast milk* protocol (P1) Age group (months)			Non-breast milk protocol (P2) Age group (months)		
	6-9	10-11	12+	5-9	10-11	12+
Water turnover %						
Mean	[19.96]	[18.60]	[17.60]	20.55	19.74	19.80
Median	[20.12]	[16.91]	[16.75]	19.41	18.38	18.48
SD	[3.94]	[6.97]	[4.32]	5.08	5.53	6.47
Upper 2.5 percentile	[26.99]	[44.57]	[31.87]	35.57	30.29	36.70
Lower 2.5 percentile	[13.88]	[10.67]	[11.67]	15.16	10.31	11.98
Water flux (L/kg/day)						
Mean	[0.11]	[0.11]	[0.10]	0.12	0.11	0.11
Median	[0.12]	[0.10]	[0.09]	0.11	0.10	0.10
SD	[0.02]	[0.04]	[0.02]	0.03	0.03	0.04
Upper 2.5 percentile	[0.15]	[0.26]	[0.18]	0.20	0.17	0.20
Lower 2.5 percentile	[0.08]	[0.06]	[0.07]	0.08	0.06	0.07
Water flux (L/day)						
Mean	[0.96]	[0.92]	[1.02]	1.06	1.08	1.19
Median	[0.93]	[0.86]	[0.95]	1.03	1.01	1.12
SD	[0.21]	[0.31]	[0.29]	0.26	0.29	0.42
Upper 2.5 percentile	[1.50]	[1.98]	[1.72]	1.78	1.65	2.34
Lower 2.5 percentile	[0.61]	[0.48]	[0.63]	0.71	0.69	0.67
Bases (unweighted)	*39*	*22*	*29*	*96*	*76*	*360*

* Breastfeeding status is denoted by any degree of breastfeeding reported at the CAPI interview; the breastfeeding status at Stage 2 was confirmed at the clinic visit.

Table J.2
Body weight (kg) of children who undertook stable isotope protocols

	Breast milk protocol* (P1) Age group (months)			Non-breast milk protocol (P2) Age group (months)		
	6-9	10-11	12+	5-9	10-11	12+
Mean	[8.4]	[8.8]	[10.5]	9.2	9.9	11.1
Median	[8.4]	[8.6]	[10.2]	9.1	9.7	11.0
SD	[1.3]	[0.9]	[2.0]	1.2	1.2	1.4
Upper 2.5 percentile	[10.2]	[10.2]	[15.9]	11.9	12.5	13.9
Lower 2.5 percentile	[6.3]	[7.6]	[7.6]	6.6	7.9	8.6
Bases (unweighted)	*39*	*22*	*29*	*96*	*76*	*360*

* by any reported degree of breastfeeding

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