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Annex B – Statistical analysis performed on the evidence retrieved from the published scientific literature for assessing the bioavailability of 5-methylfolate compared with folic acid

Summary
Dietary folate equivalents (DFE) have been defined by the US Institute of Medicine (IOM, 1998), to take into account the difference in absorption efficiency of folic acid and natural folate (NF). In practice, a conversion factor to convert the amount of folic acid into µg DFE in foods was proposed by IOM, which is the ratio between the bioavailability of folic acid and the bioavailability of natural folate. The objective of this report is to expand the DFE equation, adding the 5-methylfolate (5-MTHF) term to the NF term and the folic acid term, to obtain, if possible, an equation in the form of: µg DFE = amount of natural folate + a x amount of folic acid + b x amount of added 5-MTHF (where a is the conversion factor set by IOM and b is the conversion factor for 5-MTHF).

To reach this objective, a statistical analysis of the data collected during a systematic review undertaken by EFSA (protocol in Appendix A of the scientific opinion) was implemented. The statistical analysis aimed at comparing the bioavailability of 5-MTHF (intervention) and folic acid (control) using data on predetermined biomarkers in studies in humans consuming repeated daily doses of these two folate forms. The comparative bioavailability of 5-MTHF with respect to folic acid, i.e. the ratio of change in biomarkers in the group of subjects who received 5-MTHF vs the group who received folic acid, was used.

The below steps were followed in the statistical analysis of the experimental studies:

✓ Aggregated data have been analysed for the adult population.
✓ Descriptive and forest plots were produced to describe graphically the bioavailability in the two groups of subjects, assessed by change (end – baseline) in the biomarkers red blood cell (RBC) total folate, serum/plasma total folate (PTF) and total homocysteine (tHcy).
✓ A meta-analytical mixed-effect regression model was set up to investigate further the bioavailability (RBC folate and PTF) for adults; the model was adjusted for a set of explanatory factors (fixed effects) and a set of factors explaining the hierarchical structure in the data (random effects).

These steps are described in detail in the following sections.
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1. Introduction

The analysis conducted aimed at comparing the bioavailability of 5-MTHF to that of folic acid. The data used for the analysis (Sections 2 to 5) in adult populations were aggregated data extracted from published studies in humans consuming repeated daily doses of these folate forms (parallel design) and collected through a systematic review undertaken by EFSA.

A first analysis by visual inspection was carried out, in order to assess what factors could affect the bioavailability in the two groups, i.e. in the group consuming 5-MTHF and in that consuming folic acid. Subsequently, the aggregated data were analysed by means of appropriate meta-regression models.

The working group (WG) decided to undertake a quantitative analysis for the biomarkers RBC folate folate and PTF, and descriptive analysis for tHcy. The choice of these three biomarkers was based on previous conclusions from the NDA Panel on folate biomarkers in the opinion on dietary reference values (DRVs) for folate (EFSA NDA Panel, 2014). In particular, RBC folate is considered the most reliable biomarker of folate status which reflects well tissue folate stores. PTF is a sensitive marker of recent dietary intake, whereas tHcy, although not specific for folate status, can complement the information obtained from RBC folate and PTF.

Finally, the comparative bioavailability, assessed by the ratio of the change (end plasma/serum concentration minus baseline concentration) in biomarkers following consumption of 5-MTHF vs folic acid, was modelled.

Individual data were requested from all corresponding authors of the included studies. Following this attempt, two datasets were received (Green et al., 2013; Troesch et al., 2019) and the evolution in the biomarker values with time was evaluated. The individual data of Green et al. (2013) corresponded to 13 adults and were not further used due to the small sample size. The individual data on infants of Troesch et al. (2019), from a larger sample of individuals who consumed an infant formula with folic acid or with calcium-L-methylfolate (CaLMF), a form of 5-MTHF authorised for use in infant and follow-on formula, were initially used in an analysis that aimed at investigating whether a separate DFE conversion factor needs to be set. During the public consultation on the draft of the Scientific Opinion, EFSA was informed that the values originally denominated as RBC folate concentrations were in fact whole blood folate concentrations. As EFSA did not have access to the individual data on haematocrit, which would have allowed to calculate RBC folate concentrations from whole blood folate concentrations, the study by Troesch et al. (2019) could not be continued to be used in the assessment any longer.

2. Data pre-processing

Data were cleaned and standardised in order to be prepared for the statistical analysis. First, all parameters describing the location and variation of the biomarker values collected from the published studies were standardised (e.g. transformed uniformly in arithmetic mean and standard deviation). Furthermore, in each study, the ratio of the changes in the folate biomarker (from baseline to the end of intervention) associated with the consumption of 5-MTHF or folic acid was calculated in order to compare their effect on the biomarker (see Figure 1).
2.1. Data standardisation

The Variation Parameter type (VPT), i.e. the measure of statistical dispersion as reported in the publications, was converted into the Standard Deviation (SD), uniformly, following the rules indicated below.

1) if the VPT was a confidence interval (CI) with a 95 % significance level (95% CI), then (assuming a normal distribution of data) the SD is obtained via equation 1.

\[ SD = \frac{UL - LL}{3.92 \sqrt{n}} \]

where UL and LL are respectively the upper and lower bounds of the 95%CI and n is the sample size;

2) if the VPT was a range and the arithmetic mean is reported in the paper, then (assuming a normal distribution of data) the SD is obtained as

\[ SD = \frac{max - min}{4} \]

where \( min \) and \( max \) are respectively the upper and lower bounds of the range.

3) if the VPT was a range and the median (med) is reported in the paper, then the SD is

\[ SD = \frac{1}{\sqrt{12}} \left[ \frac{1}{4} (min - 2med + max) + (max - min)^2 \right] \]

where \( min \) and \( max \) are respectively the upper and lower bounds of the range.

4) if the VPT was a standard error (SE), then the SD is obtained as

\[ SD = \frac{SE}{\sqrt{n}} \]

where n is the sample size.

5) if the VPT was a geometric standard deviation (GSD), then the SD is obtained via equation 5.

\[ SD = \sqrt{\exp \left( \log(GSD)^2 - 1 \right) \exp(2 \log(GM) + \log(GSD)^2)} \]

where GM is the geometric mean.

6) if the VPT was a geometric confidence interval, then the SD is obtained via equation 6.

\[ SD = \left( \frac{GUL}{GLL} \right)^{\frac{1}{2 Q(0.75)}} \]
where GUL and GLL are respectively the upper and lower bounds of the geometric confidence interval and \( Q(0.75) \) is 75th percentile of a normal distribution.

Subsequently the Location Parameter Type (LPT), i.e. the measure of location or central tendency as reported in the papers, was converted into a mean, following the rules indicated below.

7) if the LPT was expressed as a median and the range is reported in the paper, then (assuming normal distribution) the mean is obtained via equation 7.

\[
\mu := \text{Mean} = \frac{\text{min} + \text{max} + 2\text{Median}}{4}
\]

where \( \text{min} \) and \( \text{max} \) are respectively the upper and lower bound of the range.

8) if the LPT was expressed as a median and the interquartile range is reported, then (assuming lognormal distribution) the mean is obtained via equation 8.

\[
\text{Mean} = \exp\left(\log(\text{med}) + \frac{1}{2}\left(\log\left(\frac{P75}{P25}\right) + 1\right)\right)
\]

Where med is the median, \( P25 \) and \( P75 \) are respectively the lower and upper bounds of the interquartile range, and \( Q(0.75) \) is 75th percentile of a normal distribution.

9) if the LPT was expressed as a geometric mean, then the mean is obtained via equation 9.

\[
\text{Mean} = \exp\left(\log(\text{GM}) + \frac{1}{2}\log(\text{GSD})^2\right)
\]

where GM is the geometric mean and GSD is the geometric standard deviation.

The standardisation formulas were applied following the Cochrane Handbook (Higgins et al., 2019).

2.2. Computation of the ratio

The variable of interest selected for the meta-regression model for aggregated data (See Section 4 of this statistical report) was the ratio of means (ROM), between the two groups, of the difference between baseline and final values of the biomarkers investigated.

First, the mean changes from baseline within each group (\( I_a \) for the intervention group receiving 5-MTHF and \( C_a \) for the control group receiving folic acid) were calculated following the equations indicated below:

\[
I_a = I_f - I_0
\]
\[ C_A = C_f - C_0 \]  \hspace{1cm} \text{Equation 11}

where \( I_0 \) and \( I_f \) are the measurements for the intervention group at baseline and at the end of the study respectively, and \( C_0 \) and \( C_f \) are the measurements for the control group at baseline and at end of the study respectively.

The corresponding SDs of the changes from baseline for the intervention and control groups, respectively, were computed following equations 12 and 13.

\[
SD(I_\Delta) = \frac{1}{n_{I_f}} \sqrt{SD(I_f)^2 + SD(I_0)^2}
\]  \hspace{1cm} \text{Equation 12}

\[
SD(C_\Delta) = \frac{1}{n_{C_f}} \sqrt{SD(C_f)^2 + SD(C_0)^2}
\]  \hspace{1cm} \text{Equation 13}

where \( n_{I_f} \) and \( n_{C_f} \) are the sample size at end of the study of the intervention and control group respectively (Higgins et al., 2019).

Subsequently, the ROM was computed according to equation 14.

\[
ROM = \frac{I_\Delta}{C_\Delta}
\]  \hspace{1cm} \text{Equation 14}

The scheme of the procedure for the ROM calculation is summarised in Figure 1.

---

**Figure 1.**
Finally, the analysis was carried out on the natural logarithm scale, due to data distribution (Fleiss, 1993). Hence, for each study, log (ROM) and the corresponding SE were computed (Friedrich et al., 2008) according to equations 15 and 16.

\[
\log(ROM) = \log\left(\frac{I_\Delta}{C_\Delta}\right)
\]

\[
SE(\log(ROM)) = \frac{1}{n_I} \left(\frac{SD(I_\Delta)}{I_\Delta}\right)^2 + \frac{1}{n_C} \left(\frac{SD(C_\Delta)}{C_\Delta}\right)^2
\]

The rationale behind the choice of the ROMs (instead of the mean differences between groups) for the analysis was to simplify the biological interpretation (Friedrich et al., 2008) of the comparative bioavailability of the folate forms (folic acid and 5-MTHF) in the two groups. The ratio of changes from baseline was calculated in order to standardise the data and normalise any possible differences between studies due to different baseline measurements (hence to control for the possible influence of baseline folate status on the response of the biomarker).

It is possible to have three different situations depending on the ROM value:

- when ROM is equal to one, the two folate forms have a similar effect on the biomarker;
- when ROM is greater than one, 5-MTHF (I) has a greater effect on the biomarker than folic acid;
- when ROM is lower than one, folic acid (C) has a greater effect on the biomarker than 5-MTHF.

### 2.3. Computation of the dose

In line with the protocol (Appendix A of the scientific opinion), studies were included in the systematic review if they had used similar doses, preferably equimolar. When, in a paper, the dose was reported both as µg and nmol, the nmol value was extracted and considered for the analysis. When reported in micrograms only, the study dose was standardised in nmol using the following molar masses: 441.4 g/mol for folic acid and 459.5 g/mol for 5-MTHF. The aim was to express doses given to the two groups in nmol across studies, in order to evaluate correctly the comparative bioavailability of 5-MTHF.
and folic acid (i.e. due to biological differences and not to different doses in µg). The doses in nmol have been rounded after the computation (rounded to the closest unit, without any decimal after the comma).

For the study by Hekmatdoost et al. (2015), non-equimolar doses were used, but the difference in the dose expressed as nmol between the two groups (2,176 nmol/day for 5-MTHF and 2,266 nmol for folic acid) was small, hence an average was computed (2,221 nmol/day).

2.4. Data cleaning

Completeness of the dataset was checked. Not all studies reported all the necessary information to conduct the analysis or compute the parameters needed for the analysis (mean, SD and sample size per group). The authors were contacted in order to provide the missing information (as foreseen in the protocol, see also Section 2 of the scientific opinion).

The following studies (and related biomarker values) were excluded from the analysis described in this report since no answer from the authors was received:

- Diefenbach et al. (2013) (tHcy): for missing location parameter at baseline for both groups;
- Khandanpour et al. (2009) (RBC folate, PTF, tHcy): for missing location parameter at end of the study for both groups;

Additional checks were made in order to identify possible outliers and other shortcomings. Accordingly, the following studies were excluded from the statistical analysis:

- Houghton et al. (2006) (RBC folate, PTF): outlier in relation to the investigated population of lactating women in Canada (where mandatory fortification of some foods takes place). The lactating women were supplemented with 906 nmol/day of 5-MTHF (416 µg/day) or folic acid (400 µg/day) during lactation, after previous supplementation of 1 mg/day folic acid during pregnancy;
- Bailey et al. (2015) (PTF): biomarker measured during the first study period of only three days;
- Sicińska et al. (2018) (PTF): as the total dose of 5-MTHF/folic acid was administered to the subjects in two daily doses and not once daily as for the other studies in adults included;
- Bayes et al. (2019) (PTF): the measurements from this single-blind study would have introduced heterogeneity into the dataset that cannot be explained, in particular as no description of the analytical method is provided in the paper;
- Akoglu et al. (2008) (tHcy): variation of parameters too high compared to other studies (potential outlier);
- de Meer et al. (2005) (tHcy): variation of parameters too high compared to other studies (potential outlier).

Furthermore, the data extracted from the following papers were not used in this statistical report:

- Venn et al. (2002) in women of childbearing age, which was a population included in the study population of men and women investigated in Venn et al. (2003): the WG decided to consider in this statistical report only the data from Venn et al. (2003);
- Lamers et al. (2004), Lamers et al. (2006) and the study 1 in Pietrzik et al. (2007) reported data on the same subjects: the WG decided to consider in this statistical report the data on RBC folate of Pietrzik et al. (2007) expressed as arithmetic mean and SD, and not those of Lamers et al. (2006) expressed as geometric mean and CI. The WG also decided to consider in this statistical report the data on PTF from Lamers et al. (2006) measured by the microbiological
method, and not those of Lamers et al. (2004) measured by a protein binding method. The data from Lamers et al. (2004) were thus only used for tHcy.

3. Exploratory analysis

Some references reported measurements (datapoints) on different biomarkers or on the same biomarker but in different populations (e.g. different age groups). Hence, the number of datapoints discussed below is higher than the number of studies. Each datapoint considered comprises a baseline and an end of study measurement. The measurements of the biomarkers at intermediate timepoints between baseline and end of study reported in some of the included papers were not taken into account in this statistical report (intermediate timepoints not available for all papers, variable study duration).

3.1. General descriptive statistics

From the systematic review process and after the exclusions listed in Section 2.5, 16 references were available for analysis. A total of 42 datapoints (i.e. 84 measurements at baseline and end of study) and 15 different biomarkers were identified (Figure 2).

![Bar plot with the number of datapoints by biomarker](image)

**Figure 2.** Bar plot with the number of datapoints (X axis) by each biomarker (Y axis) for which values were extracted from the papers.

Datapoints correspond to either measurements of different biomarkers in various papers or of the same biomarker in different population groups that were investigated in the same paper. Hence, the number of datapoints is higher than the number of studies.

The biomarkers with the highest number of datapoints were PTF and tHcy (9), followed by RBC folate (8). All the other biomarkers identified had a lower number of studies; besides their lower relevance (see introduction of this statistical report), it was not possible to investigate them further and provide any quantitative analysis.
3.2. Graphical display

3.2.1. Duration

The studies presented a high heterogeneity in study duration (Figure 3).

For RBC folate, the maximum duration was of 168 days (Venn et al., 2003; Pietrzik et al., 2007; Diefenbach et al., 2013) and the minimum of 84 days (Henderson et al., 2018).

For PTF and tHcy, the heterogeneity was higher, with a maximum duration of 196 days (Hekmatdoost et al., 2015) for both biomarkers, and a minimum of 35 days (de Meer et al., 2005) for PTF and 28 days for tHcy (Sicińska et al., 2018).

The high variability in study duration was analysed carefully in the next sections, in order to check if and how the study duration affected the bioavailability of the folate forms in the two groups (Section 4 of this statistical report).
Figure 3. Bar-plot counting number of datapoints by study duration in days for the biomarkers RBC folate (n=8), PTF (n=9) and tHcy (n=9).

Datapoints correspond to either measurements on different biomarkers or on the same biomarker in different population groups that may be investigated in the same paper. Hence, the number of datapoints is higher than the number of studies.

3.2.2. Profile plots

Profile plots were created to have a first insight into the data. The aim was to recognise any possible trend across time and to identify outliers.

3.2.2.1. Red blood cell total folate

The profile plot (Figure 4) highlights that the studies generally showed an increase in the biomarker value with time. The direction of this change (i.e. an increase) is what was expected (EFSA NDA Panel, 2014). These studies correspond to various adult populations and one infant population (Troesch et al., 2019). Of note, all studies included in this analysis and measuring RBC folate used the microbiological method.

Visual inspection shows that, in general, the two groups present a similar behaviour, but the increase in the biomarker compared to baseline in the 5-MTHF group seems to be slightly steeper than the one in the folic acid group.

Furthermore, two possible outliers can be observed from these plots:

- The study by Zappacosta et al. (2013), which is the only study in these graphs investigating a population with hyperhomocysteinemia and considered by the WG (based on the folate biomarker values) as being with severe folate deficiency. For this study, both baseline and end of the study measurements are outside the range of the other studies (about less than half of the average of RBC folate values observed in the other studies) (see also Section 3.2.3.1);

- The study by Henderson et al. (2018) shows measurements at end of the study not in line with the measurements in the other studies. See section 3.2.3.1 for additional information.

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1 The study by Troesch et al. (2019) was excluded from the assessment after the public consultation.
Profile plot: time evolution by group [RBC total folate (nmol/L)]

Figure 4. Profile plot of the biomarker RBC folate by group.

The X-axis represents the duration, while the Y-axis represents the measurements of the RBC folate in nmol/L. The baseline measurements and the final measurements are reported as points for each study (colour) and they are linked by a segment to highlight the time evolution between baseline and end only (without considering possible intermediate timepoints in the study). The shape of the points describes the status of the population, i.e. healthy (circle) or with a disease (triangle).

3.2.2.2. Serum/Plasma total folate

As for the RBC folate, the PTF profile plot (Figure 5) generally shows an increase in the biomarker with time. The direction of this change (i.e. an increase) was what was expected (EFSA NDA Panel, 2014). These studies correspond to various adult populations, including one study on women who became pregnant during the study (Hekmatdoost et al., 2015). Visual inspection shows that, in the folic acid group, studies with lower baseline measurements (de Meer et al., 2005; Lamers et al., 2006; Henderson et al., 2018) show the largest increase in biomarker, while studies with higher baseline measurements (Venn et al., 2002; de Meer et al., 2005; Lamers et al., 2006; Wright et al., 2010; Henderson et al., 2018) show a smaller increase in the biomarker (see also Section 3.2.3.).

The plot indicates two possible outliers, which, however, in the end were kept in the analyses:

- The study by Green et al. (2013), the only study that used fortified food and microencapsulated 5-MTHF (unclear if this was also the case for folic acid), shows measurements outside the range of the other studies (both measurements are about double the average of the biomarker values of the other studies at baseline or end time);
- The study by Henderson et al. (2018), as for RBC folate, shows a steeper increase in the biomarker, but the end of the study measurement is still in the range of measurements of other studies.

Profile plot: time evolution by group [serum/plasma total folate (nmol/L)]

Figure 5. Profile plot of the biomarker PTF by group.

The X-axis represents the duration, while the Y-axis represents the measurements of the PTF in nmol/L. The baseline measurements and the final measurements are reported as points for each study (colour) and they are linked by a segment to highlight the time evolution between baseline and end only (without considering possible intermediate timepoints in the study). All studies represented in this plot investigated healthy populations.

3.2.2.3. Total homocysteine

The tHcy profile plots (Figure 6) show a decrease of the biomarker with time. The direction of this change (i.e. a decrease) was what was expected (EFSA NDA Panel, 2014). These studies correspond to various adult populations, including one study on women who became pregnant during the investigation (Hekmatdoost et al., 2015). For both investigated groups, studies with higher measurements at baseline display a greater decrease (Wright et al., 2010; Zappacosta et al., 2013; Hekmatdoost et al., 2015) than studies with lower measurements at baseline (Venn et al., 2003; Lamers et al., 2004; Houghton et al., 2006). Of note, the study by Zappacosta et al. (2013) in subjects with hyperhomocysteinaemia and assessed by the WG as having severe folate deficiency, which had been identified as a possible outlier for the RBC folate profile plots, was not identified as a possible outlier in the profile plot on tHcy.

The plots identify a possible outlier, i.e. the study by Bostom et al. (2000) in subjects with a disease (haemodialysis patients with hyperhomocysteinaemia), which reports measurements outside the range of the measurements of the other studies.
As explained elsewhere (EFSA NDA Panel, 2014), tHcy is a sensitive but not a specific biomarker of folate status and function since it is influenced by other factors such as other B-vitamins (e.g. cobalamin). Either some information was available on the intake of or supplementation with other B-vitamins (Bostom et al., 2000; Houghton et al., 2006; Wright et al., 2010), or some information was available on plasma cobalamin (vitamin B12) ranging from 209 to 720 pmol/L (or more rarely on plasma pyridoxal 5-phosphate (PLP) as a biomarker of vitamin B6 status) (see appendix A of this statistical report).

**Profile plot: time evolution by group [tHcys (µmol/L)]**

![Profile plot of the biomarker tHcy by group.](image)

**Figure 6.** Profile plot of the biomarker tHcy by group.

The X-axis represents the duration, while the Y-axis represents the measurements of the tHcy in µmol/L. The baseline measurements and the final measurements are reported as points for each study (colour) and they are linked by a segment to highlight the time evolution between baseline and end only (without considering possible intermediate timepoints in the study). The shape of the points describes the status of the population, i.e healthy (circle) or with a disease (triangle).

### 3.2.3. Forest plots of change from baseline

In this section, forest plots describing the changes from baseline computed following

\[ I_A = I_f - I_0 \quad \text{Equation 10} \]

\[ C_A = C_f - C_0 \quad \text{Equation 11} \]

and Error! Reference source not found. (see Section 2.2.) are reported. They are useful to analyse the difference in the amplitude of the change from baseline between studies and to identify possible outliers.
3.2.3.1. Red blood cell total folate

The forest plot describing the change in RBC folate from baseline, by group and for all the studies is shown in Figure 7.

All the eight studies present positive changes, i.e. the RBC folate increases during the study following 5-MTHF or folic acid supplementation. All these studies corresponded to repeated-dose studies in healthy adults receiving a daily supplement, except for Green et al. (2013) in which the healthy adults investigated received daily a fortified bread roll.

In studies characterised by a smaller change from baseline values (Venn et al., 2003; Zappacosta et al., 2013), the 5-MTHF group displays a smaller change from baseline than the folic acid group (e.g. for the study by Venn et al. (2003) change of 253 nmol/L in the 5-MTHF group, smaller than the change of 275 nmol/L in the folic acid group). On the contrary, in studies characterised by a larger change from baseline values (Pietrzik et al., 2007; Henderson et al., 2018), the 5-MTHF group shows greater changes than the folic acid group (e.g. for the study by Henderson et al. (2018); change of 1,251 nmol/L in the 5-MTHF group, larger than the change of 759 nmol/L in the folic acid group).

Furthermore, studies with larger change from baseline values per group have greater differences in changes between the two groups, than studies with smaller change from baseline values. For instance, in the study by Henderson et al. (2018) mentioned above, group difference in the change of the biomarker from baseline is 492 nmol/L, while in the study by Venn et al. (2003), group difference is 22 nmol/L.

Therefore, when the changes from baseline are small, the difference in biomarker change between the two groups are small and 5-MTHF has a (slightly) lower effect on RBC folate concentrations than folic acid. On the contrary, when the changes from baseline are large, the differences in biomarker change between the two groups are more remarkable and 5-MTHF has a higher effect on RBC folate concentrations than folic acid.

Moreover, studies with the largest change from baseline values are the ones characterised by highest dose levels investigated (e.g. 2,270 nmol/day (Henderson et al., 2018); 905 nmol/day (Pietrzik et al., 2007; Diefenbach et al., 2013; Green et al., 2013), whereas, studies with the smallest change from baseline values used smaller dose levels (453 nmol/day and below (Venn et al., 2003; Wright et al., 2010; Zappacosta et al., 2013). A possible dose-response relationship is investigated in section 4.1.1.
Figure 7. Forest plot of the change from baseline for RBC folate by group.

The X-axis represents the measurements of the RBC folate in nmol/L, while the Y-axis represents the references. The points represent the change from baseline. The bar is computed adding and subtracting from that change the SD of the change from baseline. The size of the points describes the sample size of the studies and the shape of the points describes the status of the population, i.e. healthy (triangle) or with a disease (circle). For each study, the two groups are reported by colour: 5-MTHF (red) and folic acid (blue). Information on study design (duration in days and daily dose in nmol) is reported on the left side of the plot.

The WG particularly discussed the results by Zappacosta et al. (2013) and also contacted the authors for further clarification as the unit of measurements for RBC folate was missing in the original publication. One of the authors confirmed that RBC folate concentrations were presented in nmol/L. Based on experts’ judgement, the results reported for RBC folate, indicating severe folate deficiency, were considered inconsistent with those reported for tHcy. The study was thus excluded from further statistical analysis.

Based on the above-mentioned exclusion, further steps of the analysis on RBC folate data focused on the following six studies: Diefenbach et al. (2013), Green et al. (2013), Henderson et al. (2018), Pietrzik et al. (2007), Venn et al. (2003) and Wright et al. (2010). The WG decided to include the study by Henderson et al. (2018), although previously identified in Section 3.2.2.1. as a possible outlier as measurements at end of the study that were not in line with the measurements in the other studies: it was considered that this was possibly explained by the high dose of folate investigated.
3.2.3.2. Serum/plasma total folate

The forest plot describing the change in PTF from baseline, by group and for all the studies is shown in Error! Reference source not found.. All these studies corresponded to repeated-dose studies in healthy adults receiving a daily supplement, except for Green et al. (2013) in which the healthy adults investigated received daily a fortified bread roll. The behaviour of PTF is similar to RBC folate (Figure 7) and all the studies present positive changes, i.e. PTF increases during the study following 5-MTHF or folic acid supplementation.

As for RBC folate, studies characterised by a smaller change from baseline values (Venn et al., 2003; de Meer et al., 2005; Wright et al., 2010) display smaller or similar changes from baseline in the 5-MTHF group than in the group receiving folic acid (e.g. study by Green et al. (2013): change of 20 nmol/L in the 5-MTHF group, similar to the change of 22 nmol/L in the folic acid group). On the contrary, in studies characterised by a larger change from baseline (Lamers et al., 2006; Henderson et al., 2018), the 5-MTHF group shows larger changes than the folic acid group (e.g. Hekmatdoost et al. (2015): change of 40 nmol/L in the 5-MTHF group, which is larger than the change of 29 nmol/L in the folic acid group).

Furthermore, studies with a larger change from baseline per group have greater differences in changes between the two groups than studies with smaller changes from baseline values. For instance, in the study by Hekmatdoost et al. (2015), group difference in the change of the biomarker from baseline is 10 nmol/L, while in the study by Green et al. (2013) group difference is 2 nmol/L.

Hence, similarly to RBC folate, the plot shows that smaller changes from baseline correspond to a smaller difference in biomarker change between the two groups and, in this case, 5-MTHF has a smaller effect on PTF concentrations than folic acid. On the contrary, when the changes from baseline are large, the difference in biomarker change between the two groups is more remarkable and 5-MTHF has a higher effect on PTF concentrations than folic acid.

Finally, as for RBC folate, studies with the largest change from baseline values are the ones characterised by the highest dose levels investigated (Lamers et al., 2006; Diefenbach et al., 2013; Green et al., 2013; Hekmatdoost et al., 2015; Henderson et al., 2018). Studies with the smallest change from baseline values used lower doses (Venn et al., 2003; Wright et al., 2010). A possible dose-response relationship is investigated in section 4.1.2.

Initially, further steps of the analysis on PTF data focused on the following eight studies: Diefenbach et al. (2013), Green et al. (2013), Henderson et al. (2018), Hekmatdoost et al. (2015), Lamers et al. (2006), de Meer et al. (2005), Venn et al. (2003) and Wright et al. (2010). After further consideration, it was decided to exclude the study by Hekmatdoost et al. (2015) from the statistical analysis owing to the fact that the population was women who previously had ≥3 idiopathic abortions. Only women who became pregnant and did not have a further abortion were followed up. The duration of follow-up differed (depending on when women became pregnant) and was planned up to gestational week 20. As this population was substantially different from the other study populations, the WG decided to evaluate the results of this study separately and not combine it with the other studies in the dose response analysis.

The WG decided to include the studies by Green et al. (2013) and by Henderson et al. (2018) in the statistical analysis, previously identified in section 3.2.2.1. as potential outliers, because their behaviour may be explained by the high dose of folate forms investigated (Henderson et al., 2018) or the form of folate and vehicle investigated (Green et al., 2013).
3.2.3.3. Total homocysteine

The forest plot describing the change in plasma tHcy from baseline for all the studies by group is shown in Figure 9. The behaviour is opposite to RBC folate (Figure 7) and PTF (Figure 8) as expected, i.e. plasma tHcy decreases during the studies following 5-MTHF or folic acid supplementation.

No obvious trend between the biomarker results and the dose level of the folate form or the study duration is observed.

Bostom et al. (2000) show the highest change from baseline (and this baseline value was also the highest, see Figure 6) and the investigated daily dose (36,997 nmol/day, with a co-intervention of 1 mg/day vitamin cobalamin and 50 mg/day vitamin B6) is much larger than those investigated by other authors, i.e. around 16 times more than the highest dose used in the other studies (e.g. 2,270 nmol/day (Henderson et al., 2018)).
As explained in the introduction, the WG considered that the aggregated data on tHcy could not be used to quantitatively assess a possible dose-response relationship, which will be investigated in the following sections.

**Figure 9.** Forest plot of the change from baseline for tHcy by group.

The X-axis represents the measurements of the tHcy in μmol/L, while the Y-axis represents the references. The points represent the change from baseline and the bar is computed adding and subtracting the SD of the change from baseline. The size of the points describes the sample size of the studies, and the shape of the points describes the status of the population, i.e. healthy (triangle) or with a disease (circle). For each study, the two groups are reported by colour: 5-MTHF (red) and folic acid (blue). Information on study design information (duration in days and daily dose in nmol is reported on the left side of the plot. Information on intake and/or status of other B-vitamins is mentioned in Section 3.2.2.3.

4. **Meta-analyses (adult)**

Meta-regression models have been used to investigate the relationship between the ratio of the changes of RBC folate and PTF from baseline in the 5-MTHF group (numerator) vs the folic acid group (denominator) and the daily dose administered (5-MTHF or folic acid), taking into account other variables (e.g. duration), and to assess if and how the effect of 5-MTHF on biomarker responses compared to folic acid could change.
Acknowledging some uncertainties (see Section 7.3 of this statistical report), for the purpose of the statistical analysis, one assumption made was that the background intake of folate is supposed to be the same in the two groups (intervention and control).

Also, the assumption was made: the ratio of the mean changes in biomarkers (RBC folate and PTF) following 5-MTHF or folic acid consumption, respectively, represents a good proxy of the ratio that could be observed at the individual level and of the difference in bioavailability of the folate forms between intervention group (receiving 5-MTHF) and control group (receiving folic acid).

A preliminary list of factors (e.g. dose, duration) was suggested by the experts of the WG based on the biological plausibility of their influence. The possible effect of these factors was investigated by visual inspection of the scatter plots (Section 4) and was further considered in the present analysis.

A linear shape was assumed and a suitable variable transformation (i.e. logarithmic) has been tested. For the two ratios of changes in biomarkers, the models included as fixed effects the factor suggested by the WG (i.e. the daily dose) and, where appropriate and when data were available, additional factors that were assessed for their potential to explain part of the variability of the effect or to modify the ratios (i.e. modifiers). For the PTF model, an additional random component was introduced to reflect the hierarchical structure in the data (e.g. two populations, of younger and older adults, investigated in the same study (de Meer et al., 2005)).

For each biomarker, the identification of the best model, i.e. that best fitted the data, was based on three criteria considered concurrently: 1. goodness of fit assessed using the Akaike Information Criteria (AIC) (the lowest the AIC, the better the goodness of fit) (Akaike, 1974); 2. statistical significance of the parameters; 3. explained heterogeneity.

Once the best model was identified, visual inspection of the models has been used to validate it (see comments on Figures 11 and 14 in Sections 4.1.1.2 and 4.1.1.3. respectively). More thorough diagnosis of the model was prevented by the scarcity of observations.

For both RBC folate and PTF, a dose-response meta regression model was identified as the best one (Crippa and Orsini, 2016) and the results are discussed respectively in sections 4.1.1.2 and below 4.1.2.2. Subsequently, an interpretation of the model is given and the uncertainties of the models are described in Section Error! Reference source not found..

4.1.1. Ratio of changes in red blood cell total folate from baseline

4.1.1.1. Scatter plots

In order to assess potential factors to be considered in the model, scatter plots representing potential linear relationship between the calculated ratios of mean changes in RBC folate from baseline in the 5-MTHF and folic acid groups and possible covariates for which data were available for all studies (daily dose, study duration or both) are provided in Figure 10.
Figure 10. Scatter plots of ratio of mean changes in RBC folate from baseline in the 5-MTHF and folic acid groups, testing different covariates, i.e. daily dose, study duration and the interaction between daily dose and study duration, respectively.

The Y-axis represents the ratio of mean changes in RBC folate from baseline (with the 5-MTHF group as the numerator and the folic acid group as the denominator). The size of the datapoint is proportional to the sample size of the study. The X-axis represents the daily dose (nmol) in the first and third pictures, and the study duration (days) in the second picture. In the third picture, the study duration is categorised in two groups: up to 140 days (included) and strictly above 140 days. For each figure, a linear model (bold line) is drawn, showing the relationship between the ratio of mean changes and the covariate (dose or duration). The grey area around the bold line represents the 95% CI of the linear model.

The first picture shows a strong positive linear relationship between the ratio and the daily dose. This supports previous observations made when the forest plots were considered (Figure 7).

The second picture shows a negative linear relationship between the ratio and the study duration (as a continuous variable). However, this relationship is weaker than the previous one: the slope of the linear model is softer, and the 95% CI around it is wider.

The third picture shows the relationship between the ratio and the daily dose and study duration used as categorical variable (up to 140 days or strictly above 140 days). The plot shows no clear relationship, since the two regression lines seem (by visual inspection) to have similar slopes.

4.1.1.2. Linear meta-regression model

The relationship between the daily dose and the ratio of mean changes in RBC folate from baseline, after either 5-MTHF consumption (numerator) or folic acid consumption (denominator), was investigated. Also, the models having as covariate duration and daily dose-duration interaction have been tested, but the respective parameters resulted not significant and hence they have not been further investigated. A total of six data points were eligible for the analysis from the following studies identified in Section 3.2.3.1 (Venn et al., 2003; Pietrzik et al., 2007; Wright et al., 2010; Diefenbach et al., 2013; Green et al., 2013; Henderson et al., 2018).
A logarithmic transformation of the ratio was used in order to ensure that the response variable could be assumed to be normally distributed, as already mentioned in section 2.2 of this statistical report. A logarithmic transformation of the dose provided a better fit. The factors identified by the WG (dose and duration) as potential modifiers of the model and displayed in the scatter plots (section 4.1.1.1 [Error! Reference source not found.]) were tested for inclusion in the model.

The final model identified is a fixed effect dose-response meta regression model:

$$\log(y_j) = \beta_0 + \beta_1 \log(x_j) + \epsilon_j$$  \hspace{1cm} \text{Equation 15}$$

Where $y_j$ is the ratio of mean changes in RBC folate observed in the $j^{th}$ datapoint

$x_j$ is the fixed effect of the daily dose

$\epsilon_j$ is the random error indicating the distribution of the response.

To correctly interpret model coefficients, $\log(y_j) = \beta_0 + \beta_1 \log(x_j) + \epsilon_j$

Equation 15 can be rewritten, applying mathematical transformations of logarithm into exponential, as

$$y_j \sim \exp(\beta_0 \exp(\beta_1 \log(x_j))) = \exp(\beta_0) \cdot x_j^{\beta_1}$$  \hspace{1cm} \text{Equation 16}$$

Hence, substituting the intercept and slope of the model (Table 1), $\exp(-2.1176)$ is equal to 0.1203 and then the equation becomes:

$$y_j \sim 0.1203 \cdot x_j^{0.3425}$$  \hspace{1cm} \text{Equation 19}$$

The model considering a random effect associated with the studies was also assessed, but when comparing it with the model without such random effect, the latter was providing the same fit. This result indicates that there is no significant heterogeneity between the studies (see also the results of the test for residual heterogeneity in Table 1)

The model estimates and the dose-response relationship are provided in Error! Reference source not found. Error! Reference source not found. and Figure 11, respectively [Error! Reference source not found.].

Table 1: Dose-response meta-regression estimates for RBC folate, measure of heterogeneity and goodness of fit

| Test for residual heterogeneity | Model goodness of fit |
|--------------------------------|------------------------|
| QE    | df | p-val | logLik | Deviance | AICc |
| 0.6956 | 4  | 0.9519 | 3.420  | -6.8410  | 9.1590 |

| Model results |
|---------------|
| estimate | SE | zval | Pval | CI.lb | CI.ub |
| Intercept (log of the ratio): $\beta_0$ | -2.1176 | 0.33 | -6.3645 | <0.0001 | -2.7697 | -1.46 |
| Daily log dose: $\beta_1$ | 0.3425 | 0.0533 | 6.4193 | <0.0001 | 0.23 | 0.44 |

AICc: Akaike information criterion corrected, CI: confidence interval, df: degree of freedom, logLik: log-likelihood, lb: lower bound, QE: Q estimate, p-val and Pval: p-value, SE: standard error, ub: upper bound.
Figure 11. Dose-response meta-regression of the ratio of mean changes in RBC folate and the daily dose (nmol).

The red dotted line corresponds to a ratio equal to one (i.e. similar effect of 5-MTHF and folic acid on RBC folate concentrations). The size of the point is proportional to the weight of the study in the meta-regressive model.

Considering the model fit and how the points are positioned within the confidence interval of the model, it can be concluded that the model is fitting the data satisfactorily. The scarcity in the number of observations did not allow a more thorough diagnosis of the model.

4.1.1.3. Model interpretation

The meta-regression model applied $\log(y_i) = \beta_0 + \beta_1 \log(x_i) + \epsilon_i$.

Equation 15 shows that there is a significant log-linear relationship between the ratio of mean changes in RBC folate (after 5-MTHF or folic acid supplementation) and the daily dose. That implies that the bioavailability of 5-MTHF compared to folic acid changes depending on the dose.

In Figure 11, for low levels of the daily dose, the ratio described by the model is below 1, therefore indicating that folic acid has a greater effect on RBC folate concentrations than 5-MTHF.

On the contrary, for higher levels of the daily dose, the ratio described by the model is above 1, indicating that 5-MTHF has a greater effect on RBC folate concentrations than folic acid.
To be more precise, computing the range in which the CI of the model crosses the line where the ratio is equal to 1 (Figure 12), it is possible to identify three different bioavailability regions, depending on the dose:

- **Region 1**: for daily doses belonging to the range (227 nmol/day\(^2\), 382 nmol/day\(^3\)), folic acid has a slightly greater effect on RBC folate concentrations than 5-MTHF;
- **Region 2**: for daily doses belonging to the range (382 nmol/day, 614 nmol/day\(^4\)), 5-MTHF and folic acid have a similar effect on RBC folate concentrations;
- **Region 3**: for daily doses belonging to the range (614 nmol/day, 2,270 nmol/day\(^5\)), 5-MTHF has a greater effect on RBC folate concentrations than folic acid. It is observed that in this region the confidence interval of the model is wider, and therefore more uncertainty is linked to these results.

Furthermore, the minimum and maximum effects of the two forms of folate on RBC folate concentrations depend on the daily dose:

- 227 nmol corresponds to the highest daily dose investigated for which 5-MTHF has a slightly lower effect on RBC folate concentrations than folic acid: 5-MTHF has a 23% (95% CI: -14%; -32%) lower effect than folic acid.
- 906 nmol corresponding to about 400 µg\(^6\): 5-MTHF has a 23% (95% CI: 11%; 37%) higher effect than folic acid.
- 2,270 nmol, i.e. approximately the value equal to the tolerable upper intake level (UL) for folic acid, corresponds to the extreme daily dose investigated for which 5-MTHF has a greater effect on RBC folate concentrations than folic acid: 5-MTHF has a 69% (95% CI: 41%; 102%) greater effect than folic acid.

It needs to be considered that there is high uncertainty linked to this estimation: the CI is quite wide, in the third region in particular. Furthermore, in the first and second regions, respectively, there is only one point guiding the model.

\(^2\) i.e. daily dose of about 100 µg folic acid or 104 µg 5-MTHF, considering a molecular weight of folic acid of 441.4 g/mol and of 5-MTHF of 459.5 g/mol
\(^3\) i.e. daily dose of about 168 µg folic acid or 175 µg 5-MTHF
\(^4\) i.e. daily dose of about 271 µg folic acid or 282 µg 5-MTHF
\(^5\) i.e. daily dose of about 1,002 µg folic acid or 1,043 µg 5-MTHF
\(^6\) Neural tube defects have been shown to be prevented in offspring by periconceptional ingestion of 400 µg folic acid/day in the form of supplements (EFSA DRV Panel, 2014)
Figure 12. Regions of different comparative bioavailability of 5-MTHF and folic acid, according to the dose response model (RBC folate).

4.1.2. Ratio of changes in serum/plasma total folate from baseline

4.1.2.1. Scatter plots

In order to assess potential factors for the model, scatter plots representing a potential linear relationship between the calculated ratios of mean changes in PTF from baseline in the 5-MTHF and folic acid groups and possible covariates for which data were available for all studies are provided in Figure 13.

In the first picture, a strong positive linear relationship between the ratio and the daily dose is shown, as already anticipated by inspection of the forest plots (Figure 8).

The second picture shows that there is no relationship between the ratio and the study duration (as a continuous variable), since the slope is parallel to the X-axis.

In the third picture, the interaction between the daily dose and the study duration is investigated. The duration was used as a categorical variable (up to 140 days or strictly above 140 days). The plot shows no clear relationship, since the two regression lines seem (by visual inspection) to have a similar slope and intercept.
Scatter plot - Ratio of means by dose
Serum/plasma total folate

Scatter plot - Ratio of means by duration
Serum/plasma total folate
**Figure 13.** Scatterplot of ratio of mean changes in PTF from baseline in the 5-MTHF and folic acid groups, testing different covariates, respectively daily dose, study duration and the interaction between daily dose and study duration.

The Y-axis represents the ratio of mean changes in PTF from baseline (with the 5-MTHF groups as the numerator and the folic acid group as the denominator). The size of the datapoint is proportional to the sample size of the study. The X-axis represents the daily dose (nmol) in the first and third picture, the study duration (days) in the second picture. In the third picture, the study duration is categorised in two groups: up to 140 days (included) and strictly above 140 days. For each figure, a linear model (bold line) is drawn, showing the relationship between the ratio of mean changes and the covariate (dose or duration). The grey area around the bold line represents the 95% confidence interval of the linear model.

### 4.1.2.2. Linear meta-regression model

The relationship between the daily dose and the ratio of mean changes of PTF from baseline, after 5-MTHF (numerator) or folic acid consumption (denominator), was investigated. Compared to the RBC folate model, for PTF, an additional random component was introduced to reflect the hierarchical structure in the data, since de Meer et al. (2005).

Therefore, a total of eight data points (de Meer et al. (2005) provided two data points) were eligible for the analysis (Venn et al., 2003; de Meer et al., 2005; Lamers et al., 2006; Wright et al., 2010; Diefenbach et al., 2013; Green et al., 2013; Henderson et al., 2018).

Due to the nature of the ratio, a log transformation of the ratio was used, as already mentioned in section 2.2 of this statistical report. A logarithmic transformation of the dose provided a better fit.

The factors identified by the experts (dose and duration) as potential modifiers of the model and displayed in the scatter plots (see 4.1.2.1Error! Reference source not found.), were tested for inclusion in the model.

The equation for the final mixed effect dose-response meta regression model is provided above:
\[ \log(y_{jk}) = \beta_0 + \beta_1 \log(X) + \epsilon_j + \rho_k \]  

Equation 20

Where  
- \( y_{jk} \) is the ratio of mean changes in PTF observed in the \( j^{th} \) datapoint
- \( X \) is the fixed effect of the dose
- \( \epsilon_j \) is the random error indicating the distribution of the response
- \( \rho_k \) is the random effect associated to the studies

The final model indicates an expected increase in the log-ratio of around 0.2921 (95%CI: 0.17, 0.41, \( p<0.0001 \)) per each increase of log of the daily dose, with a negative estimate of the intercept (-1.8742, 95%CI: -2.67, -1.08, \( p<0.0001 \)) (Table 2). As for RBC folate, the PTF model indicates a clear dose response relationship between the ratio and the dose.

To correctly interpret the model, coefficients could be rewritten applying mathematical transformations of logarithm into exponential, as in Equation 19, becoming:

\[ y_j \sim -1.8742 \cdot x_j^{0.2921} \]  

Equation 21

Compared to RBC folate model, in the case of the PTF model, the heterogeneity is higher (see also results of the test for residual heterogeneity in Table 2 compared to Table 1).

The variance component of the random effect is \( \sigma^2 = 0.0088 \).

Results of model estimates and the display of the dose-response relationship are provided respectively in Error! Reference source not found.Error! Reference source not found. and Figure 14.

**Table 2:** Dose-response meta-regression estimates for plasma total folate, measure of heterogeneity and goodness of fit

| Test for residual heterogeneity | Model goodness of fit |
|--------------------------------|------------------------|
| QE | df | p-val | logLik | Deviance | AICc |
| 34.2445 | 6 | P<0.0001 | 1.7627 | -3.5253 | 14.475 |

**Model results**

| estimate | SE | zval | Pval | CI.lb | CI.ub |
|----------|----|------|------|-------|-------|
| Intercept (log of the ratio) : \( \beta_0 \) | -1.8742 | 0.41 | -4.5965 | <0.0001 | -2.67 | -1.08 |
| Daily log dose: \( \beta_1 \) | 0.2921 | 0.0603 | 4.8416 | <0.0001 | 0.17 | 0.41 |

AICc:
4.1.2.3. Model interpretation

As for RBC folate, the meta-regression model applied in \( \log(y_{jk}) = \beta_0 + \beta_1 \log(X) + \epsilon_j + \rho_k \)

**Equation** shows that there is a significant log-linear relationship between the ratio of the mean changes in PTF (after 5-MTHF or folic acid supplementation) and the daily dose. That implies that the bioavailability of 5-MTHF compared with folic acid changes depending on the dose.

In Figure 11, for low levels of the daily dose on a molar basis, the ratio described by the model is below 1, therefore indicating that folic acid has a higher effect on PTF concentrations than 5-MTHF.

On the contrary, for higher level of dose, the ratio described by the model is above 1, indicating that 5-MTHF has a higher effect on PTF concentrations than folic acid.

To be more precise, computing the range in which the CI of the model crosses the line where the ratio is equal to 1 (Figure 125), it is possible to identify three different regions, depending on the dose:

- **Region 1:** for daily doses belonging to the range (227 nmol/day\(^7\), 391 nmol/day\(^8\)) folic acid has a slightly higher effect on PTF concentrations than 5-MTHF;

---

\(^7\) i.e. daily dose of about 100 µg folic acid or 104 µg 5-MTHF

\(^8\) i.e. daily dose of about 173 µg folic acid or 180 µg 5-MTHF
• Region 2: for daily doses belonging to the range (391 nmol/day, 852 nmol/day⁹), 5-MTHF and folic acid have a similar effect on PTF concentrations;

• Region 3: for doses belonging to the range (852 nmol/day, 2,270 nmol/day¹⁰), 5-MTHF has a higher effect on PTF concentrations than folic acid.

The minimum and maximum effects of the two forms of folate on PTF concentrations depend on the daily dose:

• 227 nmol corresponds to the highest daily dose investigated for which 5-MTHF has a slightly lower effect on PTF concentrations than folic acid: 5-MTHF has a 25% [95% CI: -10%; -43%] lower effect than folic acid.

• 2,270 nmol (i.e. approximately the value corresponding to the UL for folic acid) corresponds to the extreme daily dose investigated for which 5-MTHF has a higher effect on PTF concentrations than folic acid: 5-MTHF has a 46% [95% CI: 25%; 71%] higher effect than folic acid.

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**Figure 15.** Regions of different comparative bioavailability of 5-MTHF and folic acid, according to the dose response model (PTF).

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⁹ i.e. daily dose of about 376 µg folic acid or 391 µg 5-MTHF

¹⁰ i.e. daily dose of about 1,002 mg folic acid or 1,043 mg 5-MTHF
5. Publication bias assessment

This section is dedicated to assessing publication bias. This has only been conducted for RBC folate studies included in the meta regression analysis shown in the previous section (Section 4), considering that it is the biomarker selected by the WG to assess bioavailability (see Section 7). A funnel plot has been made for the six studies considered in the meta-analysis to explore whether there is asymmetry in terms of effect size reported in each study (Figure 16). In addition, the Egger’s statistical test was performed to assess the asymmetry of the funnel plot, resulting in a t-value of 1.7737, considering the degrees of freedom of the test (df = 3) (p-value is 0.1742, indicating non-significant asymmetry). The results here presented, that do not suggest much publication bias, should be considered with caution due to the small number of studies included in the meta-analysis, which affect the power of the test assessing publication bias.

Figure 16. Funnel plot to assess publication bias.

The funnel plot shows three regions white (indicating probability levels of 0.9), light grey (probability of 0.95) and dark grey (probability of 0.99), full circles indicating each of the studies (including reference ID) and the regression line obtained when performing Egger’s statistical test.

6. Relationship between the bioavailability of 5-MTHF and folic acid in adults (based on the RBC folate model)

For the adults, the ratio between the bioavailability of 5-MTHF and folic acid is not a constant, but a function depending on the daily dose (nmol), as shown in Equation 19 that can also be written as:
\[
\frac{\% \text{ br}(\text{5MTHF})}{\% \text{ br}(FA)} = 0.1203 \cdot (\text{daily dose of 5MTHF in nmol})^{0.3425}.
\]

Expressed in µg (considering the following molecular mass: 1 nmol of 5-MTHF = 0.4595 µg of 5-MTHF) the equation becomes:

\[
\frac{\% \text{ br}(\text{5MTHF})}{\% \text{ br}(FA)} = 0.1570 \cdot (\text{5MTHF in } \mu g)^{0.3425}
\]

Equation 22

In case this function should be used to update the DFE equation, it needs to be multiplied by 1.7 and becomes

\[
c = 1.7 \cdot 0.1570 \cdot (\text{5MTHF})^{0.3425} = 0.2669 \cdot (\text{5MTHF})^{1.3425}
\]

Equation 23

Rounded to two decimals:

\[
c = 0.27 \cdot (\text{5MTHF})^{1.34}
\]

Equation 24

This equation is valid only for 5-MTHF values belonging to the range [104.3, 1043] micrograms.

7. Infant model

The infant data had originally been analysed separately from the ones on adults (excluded from meta-analysis models of Section 4, see also reasoning in Section 3.2.2.). Only one study regarding infants (Troesch et al., 2019) was found from the literature search undertaken by EFSA. The individual data of this study have been requested from the authors, but due to an error in the provided dataset, brought to the attention of EFSA during the public consultation on the draft scientific opinion, this study was excluded from final assessment.

8. Source of uncertainty

It is important to highlight the uncertainties behind the models analysed in Section 4, in order to interpret correctly the results of the DFE equations:

- The model is based on a low number of data points (6 for RBC folate and 8 for PTF) in adults.
- The model in adults is only valid for daily doses ranging between 227 and 2,270 nmol, hence the model does not describe the dose-response relationship outside this range since no data are available.
- It has not been possible to assess the influence of the conditions of consumption (with a meal, on an empty stomach, free choice) in the model in adults, since this information was not always reported.
- Background folate intake, which may influence the observed values of the folate biomarkers investigated, has not been considered in the analysis for adults, as it was not reported for all studies. Baseline values of RBC folate and PTF concentrations are however expected to be influenced by background folate intake and this aspect has been taken into account by considering, in the statistical analysis, the change in the biomarkers from baseline. Also, exclusion of users of folic acid supplements or foods fortified with folic acid was applied in most of the included repeated-dose studies. Users of folic acid supplements or fortified foods were included in one study on healthy subjects (Pietrzik et al., 2007). The working group acknowledged these considerations, however for the purpose of the statistical analysis, one assumption made is that the background intake of folate is supposed to be the same in the two groups (intervention and control) (see introduction of Section 4).
- Aggregated data have been used to assess the bioavailability of 5-MTHF compared to folic acid in adults. It remains uncertain whether similar conclusions would have been reached using...
individual data for all the included studies (individual data were requested to the authors but were available or provided by the authors only for a limited number of studies).

9. **Software**

Data editing, cleaning and statistical analysis were performed using R version 4.0.3 (R Core Team, 2013) and Rstudio version 1.3.1093. The data cleaning and standardisation have been carried out using ‘dplyr’ package (Hadley et al., 2021). The linear meta-regression was performed using the ‘metafor’ package (Viechtbauer, 2010). All the other plots have been realised with ‘ggplot2’ package (Wickham, 2016).
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Abbreviations

5-MTHF \((6S)-5\text{-methyltetrahydrofolic acid}\)

AIC Akaike Information Criterion

ANCOVA analysis of covariance

CaLMF calcium-L-methylolate

CI confidence interval

Df degree of freedom

DFE Dietary Folate Equivalents

DRV dietary reference value

EFSA European Food Safety Authority

FBP folate binding protein

GLL lower bounds of the geometric confidence interval

GM geometric mean

GSD geometric standard deviation

GUL upper bound of the geometric confidence interval

IOM Institute of Medicine

Lb lower bound

logLik log-likelihood

LPT location parameter type

med median

NF natural folate

PLP pyridoxal 5-phosphate

PTF serum/plasma total folate

p-val and Pval p-value

QE Q estimate

RBC red blood cell

ROM ratio of means
| Abbreviation | Description |
|--------------|-------------|
| SD           | standard deviation |
| SE           | standard error |
| tHcy         | total homocysteine |
| ub           | upper bound |
| UL           | tolerable upper intake level |
| VPT          | variation parameter type |
| WG           | Working Group |
Appendix A. tHcys values and related information on B-vitamins

Additional information to figure 6 (section 3.2.2.3), it is interesting to note the following points:

- In the study by Bostom et al. (2000) in a population with a disease, all subjects received a co-intervention of 1 mg/day vitamin cobalamin and 50 mg/day vitamin B6. Baseline mean plasma cobalamin was 667 and 720 pg/mL and plasma PLP (a biomarker of vitamin B6) was 110.1 and 112.0 nmol/L in the folic acid or 5-MTHF group respectively;

- In the study by Zappacosta et al. (2013) in a population with a disease, median plasma cobalamin is 307 and 310 pmol/L in the folic acid and 5-MTHF group respectively;

- In the study by Sicińska et al. (2017), mean baseline plasma cobalamin is 209 and 219 pmol/L in the folic acid or 5-MTHF group, respectively;

- In the study by Wright et al. (2010), average intakes of 2 mg/day riboflavin (vitamin B2), 2.3 mg/day vitamin B6 and 5.18 µg/day cobalamin (vitamin B12) were reported, i.e. higher than the respective UK reference nutrient intakes at that time;

- In the study by Henderson et al (2018), mean baseline plasma cobalamin is 456 and 371 pmol/L in the folic acid or 5-MTHF group, respectively;

- In the study by Hekmatdoost et al. (2015) in women who became pregnant during the study, mean plasma cobalamin (vitamin B12) at baseline was 234 and 240 pmol/L in the folic acid or 5-MTHF group respectively;

- In the study by Houghton et al. (2006), all subjects (lactating women) received a co-intervention of 1 mg/day vitamin B6 and 3 µg/day cobalamin (as well as ferrous fumarate);

- In the study by Lamers et al. (2004), mean baseline plasma cobalamin is 216 and 239 pmol/L in the folic acid or 5-MTHF group, respectively;

- In the study by Venn et al. (2003), mean baseline plasma cobalamin is 270 and 256 pmol/L in the folic acid or 5-MTHF group, respectively.