



Vitamin B12 deficiency in newborns: impact on individual's health status and healthcare costs.

Journal:	<i>Clinical Chemistry and Laboratory Medicine</i>
Manuscript ID	CCLM.2024.0692.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Ferraro, Simona; Vittore Buzzi Children's Hospital Lucchi, Simona; Vittore Buzzi Children's Hospital Montanari, Chiara; Vittore Buzzi Children's Hospital Magnani, Letizia; University of Milan Tosi, Martina; University of Milan, Health Sciences Biganzoli, Davide; Università degli Studi di Milano, Dipartimento di Scienze Cliniche e di Comunità Lugotti, Andrea; Vittore Buzzi Children's Hospital Cappelletti, Laura; Vittore Buzzi Children's Hospital Poli, Alessia; University of Milan Pratiffi, Elisa; Vittore Buzzi Children's Hospital Carelli, Stephana Saielli, Laura; Vittore Buzzi Children's Hospital Alberti, Luisella; Vittore Buzzi Children's Hospital Zuccotti, G.; Vittore Buzzi Children's Hospital Marsilio, Marta; University of Milan Verduci, Elvira; Vittore Buzzi Children's Hospital Cereda, Cristina; Pediatric Department, "V. Buzzi" Children's Hospital, Milan, Italy
Section/Category:	General Clinical Chemistry and Laboratory Medicine
Classifications:	10.112 Biomarker < 10.100 Plasma proteins < 10 Proteins and Enzymes, 10.214 Immunoassay < 10.200 Plasma enzymes < 10 Proteins and Enzymes, 10.344 Vitamin B12 < 10.300 Other biochemical constituents+ C229 < 10 Proteins and Enzymes, 20.600 Metabolism < 20 Pathologies, 20.705 Inborn errors < 20.700 Other diseases and conditions < 20 Pathologies, 20.709 Neurodegeneration < 20.700 Other diseases and conditions < 20 Pathologies
Keywords:	vitamin, deficiency, supplementation,, healthcare, cost, biomarker
Abstract:	Background: The identification of Vitamin B12 (B12) deficiency in the newborn may prevent neurological damage and a delay in the normal growth. In this study we characterized the incidence of B12 deficiency in newborns, the costs associated to the clinical diagnosis and management, and the relevance to optimize the use of cobalamin biomarkers during treatment follow-up. Methods: Starting from a continuous case series of 146470 screened newborns (November, 1st 2021- December, 3th 2023), the Regional

	Reference Laboratory for Neonatal Screening identified 87 newborns having altered levels of biomarkers of cobalamin metabolism measured by Newborn Screening. These subjects were confirmed with a nutritional B12 deficiency of maternal origin by performing the serum B12 measurements and plasma homocysteine (Hcy) both on the newborns and respective mothers. A cost analysis was performed to characterize the costs/year of identifying and managing B12 deficiency cases. Results: At baseline, median (interquartile range) serum B12 levels of 185.0 (142.3-246.0) ng/L and threefold increased plasma Hcy concentrations above the normal level confirmed a severe condition of deficiency in the newborns. After intramuscular B12 supplementation, serum B12 measured at the first follow up visit showed a fivefold increase, and the levels of Hcy returned to normal. From the healthcare perspective, the costs for diagnosing and managing all newborns with B12 deficiency is 188480 € /year. Conclusion: Preventing B12 depletion in newborns lowers healthcare costs and improves outcomes. Further studies are required to address the pathway to identify and treat pregnant with marginal/low B12 statusto achieve these goals.

SCHOLARONE™
Manuscripts

Please provide the requested information in the format and order indicated in the template. Refer to the author instructions below.

[TEMPLATE]

STATEMENT	TEXT
Acknowledgements	None to declare
Research ethics	This study was approved by the Local Ethics Committee LOMBARDIA 1 (protocol n # CET 132-2023 of December 6, 2023). All procedures adhered to the guidelines of the Local Ethical Committee and have been performed in accordance with the ethical standards as laid down in the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained for the treatment of anonymized data from a parent or a legal guardian.
Informed consent	Informed consent was obtained for the treatment of anonymized data from a parent or a legal guardian.
Author contributions	Simona Ferraro: conceptualization, writing the first draft; Simona Lucchi: conceptualization and performed the laboratory analysis; Chiara Montanari: involved in the collection of clinical data; Letizia Magnani: performed the cost analysis Andrea Lugotti: involved in the collection of clinical data Laura Saielli: performed the laboratory analysis; Elisa Pratiffi: performed the laboratory analysis; Davide Biganzoli: performed the statistical analysis; Alessia Poli: performed the cost analysis Martina Tosi: involved in the collection of clinical data Laura Cappeletti: performed the laboratory analysis Luisella Alberti: performed the laboratory analysis; Stephana Carelli: collaborator involved in the interpretation of the results Marta Marsilio: performed the cost analysis Elvira Verduci: involved in the interpretation of the results; Gianvincenzo Zuccotti: reviewed the final version Cristina Cereda: reviewed the final version

Competing interests	The authors state no conflict of interest
Research funding	None to declare
Data availability	The raw data can be obtained on request from the corresponding author

*We do not accept submissions that name Artificial Intelligence (AI) and/or Machine Learning Tools as credited author(s) on a manuscript because such tools cannot take responsibility for the submitted work and therefore cannot be considered as eligible authors. Where such tools or technologies are used as part of the design or methodology of a research study, their use should be clearly described in the Acknowledgments section.

[AUTHOR INSTRUCTIONS]

[MANDATORY STATEMENTS]

- Acknowledgments** If not applicable, please remove from the template.
- Research ethics** State the appropriate information, including that the study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).
If not required, please insert
- "The local Institutional Review Board deemed the study exempt from review" if the IRB specifically exempted the study from review **OR**
 - "Not applicable."
- Informed consent** If required, please insert
- "Informed consent was obtained from all individuals included in this study, or their legal guardians or wards."
- If not required, please insert
- "Not applicable."

Make sure that the information on Research ethics and Informed consent is consistent with the information in the manuscript.

- Author contributions** Insert the mandatory default statement and include more detailed information about an individual author's contribution, if applicable.
- "The author(s) have (has) accepted responsibility for the entire content of this manuscript and approved its submission."
- Competing interests** State if any author(s) has/have a conflict of interest, including the name of the author(s) and the reason, followed by "All other authors state no conflict of interest" (if applicable).
If none of the authors has a conflict of interest, please insert
- "The author(s) state(s) no conflict of interest."
- Research funding** State if your study was financially supported (add funding agency and grant number).
If not required, please insert
- "None declared."
- Data availability** If applicable, please insert
- "The raw data can be obtained on request from the corresponding author."
- If the type of study does not include raw data, please insert
- "Not applicable."

[OPTIONAL STATEMENTS]

In addition to the mandatory information, you can provide information on the aspects below by adding the statement(s) to the template. Make sure you follow the order and terminology given in the template.

| Consultant or advisory role | Role of sponsor | Employment or leadership | Honorarium | Patents | Other remuneration | Software availability | Trial registration |

<https://mc.manuscriptcentral.com/cclm>

August 19th, 2024

To the Editor and reviewers

Thank you for the thoughtful comments and suggestions which have contributed to improving the content and the readability of the paper.

Comments to the authors:

Abstract

“Conclusion: B12 supplementation of pregnant women with a low/marginal B12 status, and serum B12 retesting (every 3 months) is a valuable strategy to improve health outcomes in the newborns and lower healthcare costs.”: Lowering of healthcare cost by supplementation of pregnant women with low/marginal B12 status cannot be stated based on the results in the manuscript. Neither optimised reference values for pregnancy, nor sample taking frequency and its costs have been analysed in the present work. Please rewrite the conclusions and also the corresponding opinion in the “Discussion” section (page 14, line 10-14).

Authors’ answer:

The conclusions have been rewritten according to our evidence, both in the Abstract and Discussion, highlighting that further studies are required to address the clinical pathway to identify, treat and monitor pregnant women with marginal and low B12 status, in order to prevent B12 depletion in newborns.

Comments to the authors:

Introduction

1. line 23-25 and Supplementary Table 1: the Italian and European need defined as “average requirement” and “adequate intake” have roughly a 2-fold difference. Could you please give an explanation for this? If there is no known reason for this, please consider whether it is necessary to include a supplementary table for this information or not.

Authors' answer:

We thank the reviewer for this comment giving us the opportunity to better explain the meaning of the difference among these reference values. These explanations have been added in the Supplementary Material as well as in the Results [Chapter: 2 Relations between biomarkers and features of newborns and mothers].

For the Italian Dietary Reference Values, the Average Requirement (AR) refers to the level of nutrient intake that is sufficient to meet the requirements of 50% of healthy subjects, while the Population Reference Intake (PRI) is sufficient to meet the requirements of 97.5% of healthy subjects. Both refer to the Italian population. The EFSA Dietary Reference Values are based on surveys in different European Union countries, with different average dietary intakes. The Italian Dietary Reference Values concerning vitamin B12 intake are continuously updated and the V revision has been recently presented at the Sinu Congress (June 2024, the reference has been included). We have retained in the table the EFSA DRV since is generally considered an international reference, but we have considered the PRI for a reliable evaluation of the inadequacy of B12 intake.

Comments to the authors:

Materials and Methods:

Page 5, line 3-15: the authors are discussing how results of different B12 measurement methods could be compared since these are performed with different assays. Could you please include some information about serum homocysteine measurement methods and whether these results can be compared with each other or not? These measurements were also carried out by different non-NBS laboratories and could have theoretically different reference values.

Authors' answer: The information (i.e. CV) about the methods measuring Hcy at the birth centers has been included in the Methods (Chapter 2). The ratio between the detected level of Hcy and the cut-off recommended for each method by the manufacturer allowed to estimate an average threefold increase of the values above the normal threshold. This information has been included in the Results (Chapter 1).

Comments to the authors:

Page 5, Clinical management of B12 deficiency in the newborns: the authors state that during a second visit, all infants with B12 deficiency were administered a second dose. However Figure 2 and Figure 3 in the "Results" highlight that approx one month after the administration of a single dose of B12 the vitamin level is more than normal. Please discuss

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

on page 12 (line 8) not only a recommendation for the optimal time of retesting, but also in which cases the authors would suggest a second dose of B12.

Authors’ answer: We thank the reviewer for this comment.

The second administration is done *per* clinical protocol considering the severe depletion at baseline (other studies report mild depletion, only one study performed in India report a severe depletion treated with 3 doses). It is well known that one single dose provides a hight increase of B12 circulating levels, but this does not warrant the repletion of the body pool and overall the maintenance of the body pool overtime.

On the other hand, there are no data describing the physiological decrease of the vitamin in circulation subsequent to supplementation. This work can help clinicians to identify the population of newborns requiring a second those and the best timing to perform the second dose based on the decay of the serum B12 value. By characterizing the “kinetic” of serum B12 decrease overtime, after the supplementation, we could suggest that 3 months after the supplementation may be considered for B12 retesting in order to evaluate the body pool and avoid overtreatment. Anyway this preliminary data should be confirmed by additional studies specifically designed to evaluate the longitudinal trends of B12 levels after supplementation and possibly the dependence on the baseline value. This has been extensively added in the Discussion .

Comments to the authors:

Page 6, lines 35-39 “The caregivers’ opportunity costs reflect the monetary value of their time, calculated as the caregivers’ average hourly wage multiplied by the time spent on travel and clinical activities.”: please include the source of data that was used for the estimation of average hourly wage. Might the ethnical diversity have an impact on this average wage?

Authors’ answer:

We have included the links also in the text, sorry these were only in the Supplementary Material. We have updated these reference (the website has undergone a recent update, rendering the previous link inactive): Italian National Institute of Statistics (ISTAT). Retribuzione oraria per classe di dipendenti[Gross hourly earnings per hour paid for employee positions in euros (median)]. (2024). https://esploradati.istat.it/databrowser/#/it/dw/categories/IT1,Z0500LAB,1.0/LAB_EMPLW AGE/DCSC_RACLI/IT1,533_957_DF_DCSC_RACLI_6,1.0

The ethnical diversity may have an impact on this average wage, thus we have reported the median value for the population and this accounts for the ethnical diversity and thus is more generalizable. This observation has been included in the text.

Comments to the authors:

Page 6, lines 56-59 "...the birth rate in Lombardy Region is significantly decreasing alongside an increase in cases of B12 deficiency in newborns": if it is known, please mention the reason for this in the "Discussion" section.

Authors' answer:

"The birth rate in Lombardy Region is significantly decreasing" we have added in the text the source of data [Reference 3: Italian National Institute of Statistics (ISTAT). Nati vivi (2024).

http://dati.istat.it/Index.aspx?DataSetCode=DCIS_NATI2

The reference has been updated (the website has undergone a recent update, rendering the previous link inactive): Italian National Institute of Statistics (ISTAT). Nati serie storica - prov.

(2024). https://esploradati.istat.it/databrowser/#/it/dw/categories/IT1,POP,1.0/POP_BIRTHFERT/DCIS_NATI2/IT1,25_74_DF_DCIS_NATI2_1,1.0] alongside an increase in cases of B12 deficiency in newborns (data from RRLNS). That is to say that this latter evaluation has been reported by considering the increase of B12 deficiency cases in newborns retrieved in the reference Laboratory of Newborn screening of Lombardy region since 2021 to 2023 and projecting this increase up to 2028. Only the decrease of birth rate is known, but the increase of B12 deficiency in newborns is not known, and this result may be important if referred to a population which is prevalently not Caucasian. In the Discussion we have introduced this suggestion supported by the literature.

Comments to the authors:

Results:

Page 8, line 57: "The estimated B12 intake during pregnancy was 2.44 (1.35-3.09) mcg/die": how did you estimate this? Please include this in the "Materials and methods" section.

Authors' answer: We thank the reviewer for this comment, enabling us to add the information in the Materials and Methods section of the manuscript (Chapter 3).

1
2
3
4
5
6
7
8
9
Comments to the authors:

10
11
12
13
14
Page 10, lines 42-50: what factors are contributing to the significant growth of costs in the future (until 2028)? Please include this in the text and if necessary also refer to it in the “Materials and methods”.

15
16
Authors’ answer:

17
18
19
20
21
22
23
24
The figure 3 in the supplementary Material reports the projections of the increase of costs due to the costs of the increased cases of B12 deficiency cases in newborns, adjusted for the decrease of newborns. We have added the following test in the Results when explaining this figure showing the increase of the costs (Chapter : **4 Costs of the analysis and management**).

25
26
27
28
29
30
31
32
33
34
35
36
37
38
The growth of costs from 2024 to 2028 has been predicted by accounting for the cost trend analysis from 2021 to 2023. The estimation was made by estimating the average annual increase in costs, which is attributed to the growing number of newborns managed for B12 deficiency. The use of the cost trend referred to the three previous years considered the most reliable method for producing accurate predictions. The average annual cost increase is approximately € 88795 (calculated as the average of the cost increases from 2021-2022: € 93,217, and from 2022-2023: € 84,374; see Supplementary Table 6).

39
40
Comments to the authors:

41
42
43
44
45
Figure 3 figure legend: It is not clear: did patients whose B12 levels were measured at more than 50 days after initiation of supplementation receive a single dose of supplementation or two doses?

46
47
Please include this in the figure legend.

48
49
50
51
Authors’ answer: Thanks for this recommendation, the kinetic refers to the B12 values after the first dose (included in the legend to the figure).

52
53
54
55
56
57
58
59
60

Comments to the authors:

Table 1: This table shows several metabolites, however the main text states that only some of them have significant importance when considering B12 deficiency. Please explain to the reader the reason why all of these primary metabolites are included in the table. If not relevant, please do not include them (neither in Table 1 nor in the corresponding supplementary table).

Authors' answer: We have retained in the table only the biomarkers determined in the NBS which are related to B12 metabolism. This has been specified in the text and table. The others that are determined by NBS but are not correlated to cobalamin metabolism have been removed. This also from the Supplementary material.

Comments to the authors:

Table 2: How do you explain the decrease in haemoglobin concentration in the post-treatment group? Please include it in the "Discussion".

Authors' answer: We thank the reviewer for this comment that give us the opportunity to better explain the meaning of the different haemoglobin concentration; as reported in a review by Jorgensen JM et al. haemoglobin concentration is dynamic throughout the life stages. Specifically, a physiological reduction in haemoglobin concentrations occurs after birth, primarily due to increased hemolysis and then to a lower rate of erythropoiesis. (Reference :Josh M Jorgensen, Mayra Crespo-Bellido, Kathryn G Dewey Variation in hemoglobin across the life cycle and between males and females, Ann N Y Acad Sci. 2019 Aug;1450(1):105-125. doi: 10.1111/nyas.14096.)

We confirm we have added this comment in the discussion and the relative reference.

Comments to the authors:

Discussion:

Page 13, lines 14-19: the present study truly explores the association of the deficiency with maternal eating habits, but did not make any comparisons with the eating habits of mothers from non-vitamin deficient newborns. The article does neither state any correlation with a certain eating habit and B12 deficiency, nor examined the prevalence of eating habits compared to the healthy population. Therefore the following statement is not based on the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

results of the current work: “ ...highlighting the relevance of early nutritional care of the mother from conception onwards...” Please rewrite this.

Authors’ answer: We have considered the lack of a control group a limitation and this has been specified in the discussion, revising the sentence that has been highlighted.

For Review Only

Vitamin B12 deficiency in newborns: impact on individual's health status and healthcare costs.

Simona Ferraro^{1,2^§}, Simona Lucchi^{1^}, Chiara Montanari^{2,3^}, Letizia Magnani^{4^}, Martina Tosi^{2,5}, Davide Biganzoli¹, Andrea Lugotti², Laura Cappelletti¹, Alessia Poli⁴, Elisa Pratiffi¹, Stephana Carelli^{1,6}, Laura Saielli¹, Luisella Alberti¹, Gianvincenzo Zuccotti^{2,3}, Marta Marsilio⁴, Elvira Verduci^{2,5,7^^}, Cristina Cereda^{1,2^^}

[^] Equal contribution

^{^^} Equal contribution as co-senior authors

¹Center of Functional Genomics and Rare Diseases, Buzzi Children's Hospital, Milan, Italy

² Department of Pediatrics, Buzzi Children's Hospital, Milan, Italy

³ Department of Biomedical and Clinical Science, University of Milan, 20157 Milan, Italy

⁴Department of Economics, Management, and Quantitative Methods, University of Milan, 20157 Milan, Italy

⁵Department of Health Sciences, University of Milan, 20146 Milan, Italy

⁶ Pediatric Clinical Research Center "Romeo ed Enrica Invernizzi", University of Milan, Italy

⁷Metabolic Disease Unit, Buzzi Children's Hospital, Milan, Italy

§Address correspondence to:

Simona Ferraro

e-mail: simona.ferraro@asst-fbf-sacco.it

At: Center of Functional Genomics and Rare diseases Dept. of Pediatrics Buzzi Children's Hospital, Milan, Italy

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Abstract

Background: The identification of Vitamin B12 (B12) deficiency in the newborn may prevent neurological damage and a delay in the normal growth. In this study we characterized the incidence of B12 deficiency in newborns, the costs associated to the clinical diagnosis and management, and the relevance to optimize the use of cobalamin biomarkers during treatment follow-up.

Methods: Starting from a continuous case series of 146470 screened newborns (November, 1st 2021- December, 3th 2023), the Regional Reference Laboratory for Neonatal Screening identified 87 newborns having altered levels of biomarkers of cobalamin metabolism measured by Newborn Screening. These subjects were confirmed with a nutritional B12 deficiency of maternal origin by performing the serum B12 measurements and plasma homocysteine (Hcy) both on the newborns and respective mothers. A cost analysis was performed to characterize the costs/year of identifying and managing B12 deficiency cases.

Results: At baseline, median (interquartile range) serum B12 levels of 185.0 (142.3-246.0) ng/L and threefold increased plasma Hcy concentrations above the normal level confirmed a severe condition of deficiency in the newborns. After intramuscular B12 supplementation, serum B12 measured at the first follow up visit showed a fivefold increase, and the levels of Hcy returned to normal. From the healthcare perspective, the costs for diagnosing and managing all newborns with B12 deficiency is 188480 € /year.

Conclusion: Preventing B12 depletion in newborns lowers healthcare costs and likely improves their health outcomes. Further studies are however required to address the clinical pathway to identify, treat and monitor pregnant women with marginal and low B12 status, in order to achieve these goals.

Keywords: vitamin, deficiency, supplementation, healthcare, cost, biomarkers, immunoassay.

Abbreviations: Vitamin B12 (B12), Homocysteine (Hcy), Newborn screening (NBS), Methionine (Met), Flow injection tandem mass spectrometry (FIA-MS/MS), Methylmalonic acid (MMA), Propionylcarnitine (C3), Collaborative Laboratory Integrated Reports (CLIR – Mayo Clinic), Regional Healthcare Service (RHS), Chemiluminescence (CLIA), Interquartile range (IQR), Dried blood spot samples (DBS), Population reference intake (PRI), Mean corpuscular volume (MCV), Between-subject biological variability (CVg), within-subject biological variability (CVw).

Introduction

Vitamin B12 (B12) deficiency represents a treatable condition in the newborns and their mothers. An early diagnosis in the neonatal period is required to prevent irreversible neurological damage and a delay in the normal growth [1]. Newborn screening (NBS) programme encompasses biomarkers-based risk algorithms to identify newborns with rare genetic defects, including those of cobalamin metabolism (methylmalonic acidemia, propionic acidemia or remethylation defects). The screening process enables also the detection of B12 nutritional deficiency [2]. Low dietary intake, malabsorption disorders, unhealthy eating habits or dietary patterns limiting/excluding foods from animal origin (e.g. vegetarians or vegans), are associated to a high risk of developing B12 deficiency, in absence of adequate supplementation [3,4]. The nutritional requirements of B12 during pregnancy and breastfeeding have been recently revised in the Italian population [5] by considering that B12 intake is a crucial issue for fetus and newborn development (Supplementary Table 1). Indeed, B12 acts as co-factor and co-substrate of enzymes involved in several different metabolic pathways, including the synthesis of methionine (Met) from homocysteine (Hcy) and of other methylated compounds [6,7].

In this prospective study, we have considered a continuous case series of newborns, resulting with altered biomarkers of cobalamin metabolism by NBS and confirmed to carry B12 nutritional deficiency of maternal origin by serum B12 lowest levels. The first aim of this study is to the characterize the current incidence of B12 deficiency in newborns and the costs associated to clinical diagnosis and management, in order to characterize possible preventive actions. The second aim is to critically appraise some issues related to the measurement of cobalamin related biomarkers and to optimize their monitoring as surrogate index of treatment effect.

Material and Methods

1. Enrollment of patients

From 1st November 2021 to 3th December 2023, starting from a population of 146470 screened newborns, the Regional Reference Laboratory for Neonatal Screening identified 87 subjects having altered levels of biomarkers related to cobalamin metabolism suggestive for B12 deficiency of maternal origin. This was confirmed by performing the serum B12 measurement both on the newborns and their mothers. All the newborns referred to the Metabolic Diseases and Clinical Nutrition Service of the Buzzi Children Hospital for the treatment of the vitamin deficiency

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

(Supplementary Figure 1). This study was approved by the Local Ethics Committee LOMBARDIA 1 (protocol n # CET 132-2023 of December 6, 2023). All procedures adhered to the guidelines of the Local Ethical Committee and have been performed in accordance with the ethical standards as laid down in the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained for the treatment of anonymized data from a parent or a legal guardian.

2. *Newborn Screening*

Dried blood spot samples (DBS) were obtained from newborns between 48 and 72 hours of life collecting a drop of blood from heel puncture on a Whatman 903 filter paper (Guthrie card) and drying it at room temperature for several hours, before sending it to the Regional Reference Laboratory for Neonatal Screening for the analysis. On the DBS the concentrations of 36 carnitines, 14 amino acids, one keto acid, 2 nucleosides (Adenosine and 2'-Deossiadenosine) and 4 lysophospholipids were measured by flow injection tandem mass spectrometry (FIA-MS/MS) by Neobase 2 Kit (Revvity Italia, www.revvity.com/product/neobase-2-non-derivatized-msms-kit-3044-001u).

The selection of patients at risk of possible inherited metabolic disorders by NBS is driven by a web-based application (Collaborative Laboratory Integrated Reports (CLIR – Mayo Clinic)) allowing to identify anomalous concentrations of the measured markers, by considering reference thresholds which are updated in real time, and to assess the risk of inborn errors of metabolism [8-9].

Propionylcarnitine (C3) and Met are re-tested on the same DBS of newborns identified at risk of possible methylmalonic acidemia, propionic acidemia or remethylation defects. Furthermore, additional measurements (second tier test) of methylmalonic acid, Hcy, methylcitric acid, and 3-hydroxypropionic acid are performed by Liquid Chromatography-MS/MS (Sciex triple-quad SCIEX, <https://www.sciex.com>). Increased levels of methylmalonic acid (MMA) and/or Hcy, along with normal levels of methylcitric acid and 3-hydroxypropionic acid, identify newborns suspected for B12 nutritional deficiency. Thereafter, a second DBS is requested to the birth center, to confirm the results of the altered markers. Simultaneously, the measurements of B12 and Hcy concentrations on serum and plasma samples both from the newborns and from their mothers are performed. If the results on

DBS are confirmed along with the evidence of serum B12 levels decrease and plasma Hcy levels increase, in the newborn and the mother the nutritional deficiency is confirmed.

Noteworthy, the measurement of serum B12 obtained for overall case series from the birth center is performed by different laboratories and thus by different methods. We retained only data related to the measurement of total B12 and retrieved the information about the chemiluminescent assays used (Elettrochemiluminescence (ECLIA) Roche Cobas, Chemiluminescence (CLIA) Beckman Coulter, CLIA Unicel DxI, CLIA Siemens Atellica, Chemiluminescence Microparticle Assay (CMIA) Abbott Alinity). We retrieved the inter-methods coefficient of variability (CV), by the External Quality Assurance Services (EQAS) programs of Lombardy Region, for a mean (SD) value of B12 of 219.3(36.7) ng/L (next to the threshold value for a likely risk of B12 deficiency) which amounted to 16.7%.

The measurements of total B12 during the clinical follow-up (including pre-treatment and post-treatment levels) were performed by ECLIA Roche Cobas.

Also the measurement of plasma Hcy obtained for overall case series from the birth center is performed by different laboratories and thus by different methods (Enzymatic Assay Roche/Hitachi cobas c systems, CLIA Siemens Atellica, CMIA Abbott Alinity, Enzymatic assay Beckman Coulter AU).

We retrieved the inter-methods CV, by the EQAS programs of Lombardy Region, for a mean (SD) value of plasma Hcy of 24.4 (5.4) $\mu\text{mol/L}$ (the one value next to the values detected by the birth centers) which amounted to 21.9%.

The measurements of plasma Hcy during the clinical follow-up (including pre-treatment and post-treatment levels) were performed by CMIA Abbott Alinity .

3. Clinical management of B12 deficiency in the newborns

Newborns are scheduled for at least two visits: the first one between the 20th and 30th day of life, the second one at 2 months of age. Both visits include: 1) the evaluation of haematological and biochemical tests (serum B12 and folate, complete blood count, peripheral blood smear, plasma Hcy, plasma and urinary methylmalonic acid (MMA), plasma acylcarnitines, and amino acids), 2) specialist consultation with dietary and clinical nutrition counselling, and 3) neurological assessment.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

At the first visit the newborn is treated with intramuscular B12 supplementation (hydroxocobalamin 1000 mcg). Oral folic acid supplementation at 0.4 mg per day is also recommended until the next check-up. For the mother the same biochemical and haematological tests are available and the intramuscular B12 supplementation (hydroxocobalamin 1000 mcg) and folic acid supplementation (0.4 mg per day) are recommended until the next check-up. At the second visit intramuscular vitamin B12 supplementation is repeated (hydroxocobalamin 1000 mcg) in the newborn, recommending to continue the oral supplementation of B12 and folic acid, accounting for the biochemical results and the type of feeding. Furthermore, food intakes of mothers during pregnancy and lactation were retrospectively recorded by means of a dietary recall carried out by an expert dietician. The recall concerned eating habits, from breakfast to after dinner meals, investigating the quality, quantity and frequency of food consumption. Quantification and analysis of energy and nutrients intakes, as well as nutrient composition, were performed by MètaDieta® software (<https://www.metadieta.it/software>). The estimated B12 intake during pregnancy was compared to the National Recommended Energy and Nutrient Intake Levels population reference intake (PRI)(Supplementary table 1).

4. Total cost of the analysis and management

To evaluate the economic impact of diagnosis and management of B12 deficiency of newborns a cost analysis was carried out. Many sources of information reported in the Supplementary Table 2 (e.g., general demographic databases, clinician’s interviews, hospital management control system), were interrogated through the analysis, to achieve an evidence-based output. We adopted a multiple perspective approach that includes the Regional Healthcare Service (RHS), which is in charge of all the healthcare-related costs in the Italian National Healthcare Service, and the caregivers. The clinician’s report that the caregiver is almost always present, spends a part of time on travel and incurs the direct costs associated with travel. We identified the direct and indirect cost of both the RHS and caregivers (Supplementary Table 2). Specifically, indirect costs are measured as opportunity costs, representing the potential benefits lost by both the RHS and the caregiver when one alternative is chosen over another. For the RHS, this benefit is quantified as the cost of medical time, whereas for the caregiver, it is measured as the average cost of working time.

The costs of the diagnosis and management of B12 deficiency in newborns, have been estimated for 3 consecutive years (2021, 2022, 2023), by identifying the costs related to 3 phases: confirmation of

B12 deficiency, treatment initiation and follow up (Supplementary Figure 2). The costs of the activities in each phase have been estimated.

In particular the costs are estimated and organized by the point of view (RHS and caregiver), and then further partitioned into direct costs and opportunity costs (considered as the cost of time) for each specific case (Supplementary Table 3). From the RHS standpoint, the direct costs are associated with the clinical activities outlined in the aforementioned phases, including laboratory tests and B12 supplementation, valued according to the RHS reimbursement levels. The opportunity costs reflect the monetary value of healthcare professionals' time (laboratory professionals, metabolic specialists, neurologists, dietitians, and nurses). This time is quantified by interviews with professionals. From the caregiver's standpoint, the direct costs are related to travel (car consumption) to attend the first and second visits. The caregivers' opportunity costs reflect the monetary value of their time, calculated as the caregivers' average hourly wage multiplied by the time spent on travel and clinical activities [10, 11]. Noteworthy, the ethnical diversity may have an impact on this average wage, thus it has been considered the median value for the population which accounts for the ethnical diversity, resulting more generalizable.

5. Costs sensitivity analysis

The cost calculation maintains a conservative approach (e.g. the lowest possible cost items were selected for current clinical practice). This approach favors the least expensive scenario alternatives, for example minimizing travel costs and including the presence of a second caregiver only when required. The analysis was repeated over three years (2021, 2022, 2023) to verify its consistency and trend. Finally, the results were projected over a four-year time horizon. To ensure the results are not overestimated, the trend in the number of newborns and the sample size of newborns with vitamin B12 deficiency was analyzed. We used 2023 data as estimates for future years, despite the birth rate in Lombardy Region is significantly decreasing [12] alongside an increase in cases of B12 deficiency in newborns (data from Regional Reference Laboratory for Neonatal Screening).

6. Statistical Methods

Continuous variables were summarized as median and interquartile range (IQR). Linear regression and quantile regression were used to measure the association between serum B12 levels at baseline

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

and cobalamin metabolism related biomarkers measured at NBS, adjusting for mother’s diet, ethnicity, gestational age and B12 intake. Possible interactions were considered.

A paired t-test was used to assess the statistical significance of the post- treatment biomarkers changes (serum B12, plasma C3 and Hcy, plasma and urine MMA, hemoglobin, mean corpuscular volume (MCV) and neutrophil count).

A linear modeling approach was employed to characterize the relationship between post-treatment B12 levels and the time elapsed from treatment initiation. LOESS smoother function was used to visualize this relationship.

Statistical analyses were conducted by using R programming language (R Core Team (2024); R version 4.3.3). Plots and images were produced with ggplot2 and grain [13].

Results

1 Biomarkers- based diagnostic pathway

Eighty-seven patients were preliminary identified at low risk for Methylmalonic Aciduria (93.1% of cases), or Propionic Aciduria or Remethylation defects by R4/CLIR [8,9]. In these patients a close scrutiny of the biomarkers levels for disorders of propionate metabolism was performed (i.e. C3, 3-OH-Palmitoylcarnitine (C16:OH), Met) and the measurement of MMA and Hcy was added on the same DBS. Median (IQR) concentrations of the biomarkers **related to cobalamin metabolism** and measured on the two serial DBS, are reported in Table 1. The threshold values, adjusted for weight and age of the biomarkers are available in the Supplementary material (Supplementary Table 4). The two DBS were obtained at a median (IQR) 50 (49- 58) h and 312 (220-357) h from birth respectively. In our case series, in median a marginal increase was observed for Hcy and for MMA on the first and second DBS, respectively. The second DBS was required to the birth center to re-test the previous evaluated markers, simultaneously to the measurement of B12 and Hcy on serum and plasma samples respectively.

Median (IQR) of serum B12 measurements at baseline in newborns was 185.0 (142.3-246.0) ng/L. Seven newborns were excluded from the case series since only the measurement of holotranscobalamin was available. By considering that the laboratories declared different total B12 methods, and these are poorly harmonized [1], we reported the ratio between each measured value and the Lower Reference Level (LRL) declared by the method. The median of these estimates was 0.96 (0.75-1.26) and this suggested that the case series was characterized by a severe condition of

B12 depletion. Noteworthy, more than 50% of newborns were below the LRL. Median plasma levels (IQR) of Hcy were 16.6 (12.6-21.6) $\mu\text{mol/L}$, and the ratio between each value and the Upper Reference Level recommended by the manufacturer, revealed that in average Hcy is ~ threefold increased above the normal level.

The quantile regression model performed to investigate the association of biomarkers' levels detected at NBS on both DBS, with B12 levels at baseline, adjusting for gestational age, diet regimen of the mother and ethnicity showed only a marginal association between the increase of C16:OH and B12 levels (coefficient estimate: -3.7; Lower-Upper 95th percentile CI: -6.9,2.8; Supplementary Table 5).

2 Relations between biomarkers and features of newborns and mothers

In overall case series the gestational age was in median (IQR) 39 (38-40) weeks, 14.9% of newborns were pre-term (7.5% is the annual estimate obtained from overall population of newborns), and 44% were male. At birth, weight and height were 3180 (2760-3440) g and 50 (48-51) cm respectively, the head circumference was 34 (33-35) cm. It was reported that 82.8% of newborns received an Apgar score in the range 9-10 at 1 and 5 minutes indicating that they are adjusting well to extrauterine life. Fifty-seven% of newborns were exclusively breastfed, 28% received mixed feeding and the remaining only infant formula.

The mothers had a median age of 32 (29-36) years, most of them were Asiatic (46%) and Caucasian (42.8%) and the last group (11.2%) includes Latinos and Africans. The case series partitioned for ethnicity and type of diet (i.e. vegan, vegetarian) is reported in Figure 1. Maternal B12 and Hcy levels measured in the mothers at the birth center simultaneously to newborns were in median 222.0 (166.0-307.0) ng/L and 12.6 (10.3-15.4) $\mu\text{mol/L}$. B12 levels were next to the LRL and Hcy was two-fold higher than the Upper reference Level (URL). These values confirmed the severe B12 deficiency in the mothers as it was observed in their newborns.

The estimated B12 intake during pregnancy was 2.44 (1.35-3.09) mcg/die, and for 59% of the mothers it was judged to be inadequate since below the National Recommended Energy and Nutrient Intake Levels population reference intake (PRI) level of 2.6 mcg/die for pregnancy (a level of nutrient intake which is sufficient to meet the requirements of 97.5% of healthy pregnant subjects, Supplementary Table 1). We used PRI to provide a reliable assessment since it continuously revised whereas the European Food Safety Authority (EFSA) Dietary Reference Values are based on surveys performed in different European Union countries, and thus based on very different average dietary intakes (Supplementary Table 1).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

In Figure 1 we have reported median B12 levels within each partition (diet regimen and ethnicity) both for mothers and their newborns. There was no statistically significant difference between B12 levels observed in the different ethnicities and dietary regimens. Furthermore, there was no correlation between baseline maternal and newborn B12 levels measured at the laboratory of the birth center. These results were however expected by considering the narrow range of B12 levels in both case series.

Folate status was evaluated both on mothers and newborns in 82% of the case series; 17% of samples obtained in newborns resulted with interference due to haemolysis.

Median levels in mothers and their newborns were 6.3 µg/L (4.0-11.5 with a lower reference level (LRL) recommended in pregnancy/ lactation of 8.7 µg/L) and 20 (18.8-20; LRL=1.3) µg/L. Fifty-eight % of the mothers declared folic acid supplementation during pregnancy.

3 Post-treatment biomarkers changes in newborns

At the first visit all data related to biomarkers measured before treatment were available for 72.4% of newborns. Indeed, data from 27.6% of newborns were not considered since treated before the referral to the metabolic center (n=6), or lost at follow up (n=12) or reported only holotranscobalamin values (n=6). At the second visit, in median 33 (25-40) days after treatment initiation, the biomarkers measurements were available in 73 newborns since 11% of the patients was lost at follow up. We investigated the changes of serum B12, plasma C3, plasma Hcy, plasma and urinary MMA, haemoglobin concentration, mean corpuscular volume (MCV) and neutrophils count after B12 treatment (Table 2).

Neutrophils count was considered since morphological and quantitative neutrophil abnormalities were suspected, being common in the megaloblastic anemias associated to B12 deficiency [14]. Anyway, the detected counts were within the normal reference range and did not change after B12 supplementation.

Excepted for neutrophils, for all biomarkers we observed a significant decrease ($p<0.001$; Table 2, Figure 2) simultaneous to B12 increased values. By considering the association between marker changes and the time elapsed from treatment initiation, this appeared evident mainly for B12 levels (Figure 2). Thus, in Figure 3 we showed that B12 levels measured post treatment decrease with the increase of the time elapsed from treatment, (negative relationship, $p<0.000001$) by characterizing a “kinetic” of decrease. Serum B12 retesting after 3 months from supplementation may be considered to evaluate the body pool and avoid overtreatment.

4 Costs of the analysis and management

The cost analysis of the individual case results in 3578.6 € for the entire clinical practice of a newborn with B12 deficiency, rising to 3875.75 € for a preterm newborn. Breaking down the costs by perspective, the total costs for the RHS is 3253.16 €, increasing to 3550.31 € for the preterm newborn. The total cost for the caregiver is 325.44 €.

Supplementary Figure 3 and Supplementary Table 6 displays the costs for all cases throughout the years on a medium-term perspective. The findings pertain specifically to patients who received treatment from the Metabolic Diseases and Clinical Nutrition Service at Buzzi Children Hospital. The sample size is 29 for the year 2021, 52 for the year 2022, and 73 for the year 2023.

The increase in costs is attributed to the estimated average increase in costs over the years, driven by the observed trend of a rising number of newborns with B12 deficiency despite a decrease in the birth rate in Lombardy Region.

On average over 2021, 2022 and 2023, the analysis shows a result of 207735 € for all cases of newborns with B12 deficiency. Nevertheless, significant growth is anticipated over the years, with an average expected value over the medium term (2021-2028) of 428803 € per year.

Figure 4 presents the same data of the Supplementary Figure 3, providing details of the costs for both the RHS and the caregiver.

The growth of costs from 2024 to 2028 has been predicted by accounting for the cost trend analysis from 2021 to 2023. The estimation was made by calculating the average annual increase in costs, which is attributed to the growing number of newborns managed for B12 deficiency. The use of the cost trend, referred to the three previous years, is considered the most reliable method for producing accurate predictions. The average annual cost increase is approximately € 88795 (calculated as the average of the cost increases from 2021 (29 newborns with B12 deficiency) to 2022 (52 newborns): 93217 €, and from 2022 to 2023 (73 newborns): 84374 €; see Supplementary Table 6).

From the RHS perspective, on average over 2021, 2022 and 2023, the analysis shows a result of 188480 € for all cases of newborns with B12 deficiency. Significant growth is expected over the years, with an average value over the medium term (2021-2028) of 389089 € per year. From the caregiver perspective, the analysis (2021-2023) results in 19255 € for the case series of newborns

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

with B12 deficiency. A growth over the years is expected, with an average value over the medium term (2021-2028) of 39714 € per year.

Discussion

The maternal B12 deficiency is transferred to the newborn and may be incidentally detected at NBS, during the rule out of inherited cobalamin disorders (i.e. Methylmalonic Aciduria, Propionic Aciduria or Remethylation defects) [2]. B12 depletion is asymptomatic in the mother and in the newborn. Only in severe cases, symptoms as feeding difficulties, hypotonia, tremor, apneas and seizures, may appear between 1 and 6 months of age [15-17]. The early diagnosis of B12 deficiency which may be reverted by supplementation of B12 prevents from the developmental delay [16,17]. According to our results, NBS biomarkers included in a complex diagnostic pathway, encompassing risk algorithms and reflex testing used to identify individuals with a suspected inborn error of cobalamin metabolism, enable the characterization of a severe condition of B12 nutritional deficiency in the newborns. Indeed, more than 55% of the selected subjects show serum B12 levels below the LRL of 200 ng/L, adopted by most studies as conventional cut-off value for identifying a severe deficiency, by assuming a good comparability between assays [1]. Anyway, B12 assays are poorly harmonized [18] and by reporting the ratio between B12 serum level and the LRL declared by the method, we found that 52.5% of newborns were under the threshold value defining the severe deficiency. By applying reverse cascade screening (RCS) to the mothers [2], the severe B12 depletion in the newborns was associated to the nutritional deficiency of the mothers who showed B12 levels of ~220 ng/L, and ~ two-fold increased levels of Hcy above the URL. As expected, within this case series of subjects with a severe depletion, characterized by a narrow range of low B12 concentrations, we do not find any relationship between NBS markers and serum B12 levels. In other words, greater increases of C3, C16:OH, and decreases of Met did not predict lower B12 results. For the same reason, we do not find either a correlation between B12 levels obtained by the mothers and their newborns, or an association between B12 levels measured in both case series and ethnicity and diet regimen of the mother. Apart from the narrow range of B12 detected in both case series, we should consider the between-subject biological variability (CVg) of B12 which is high and far higher than the within-subject biological variability (CVw). Accordingly, only monitoring serial individual changes is effective to classify patients according to their B12 status [1]. Thus, pre-treatment B12 levels reported at the first visit, performed in median 18 days after the conclusion of

the diagnostic pathway by the NBS center, were slightly higher than those measured at the birth center (the median ratio of B12 to the LRL changed from 0.96 to 1.05), since 43% of newborns received infant formula or mixed feeding. Infant formula mainly provides the infant with free B12 [19]. By considering those markers whose changes are associated to cobalamin metabolism, at pre-treatment evaluation we observed a two-fold increase of Hcy and of pMMA levels and a fourteen-fold rise of uMMA above the threshold values. Substantially, hematological parameters were within the reference ranges. In median, 5 weeks after the treatment, we recorded a clinically relevant increase of B12 vitamin (~5.5 folds above the LRL) and a significant fall of cobalamin metabolism related biomarkers ($p < 0.001$). Plasma Hcy and MMA levels decreased, resulting largely within the URL. In particular, uMMA levels decreased of ~10 folds with respect to the baseline. A significant reduction of MCV and of haemoglobin concentrations, remaining however within the normal reference values, were also recorded. This was expected in this population, since haemoglobin concentration is dynamic throughout the first weeks of life, and a physiological reduction occurs after birth, primarily due to increased haemolysis and then to a lower rate of erythropoiesis [20]. Accordingly, the increase of age (weeks) may be considered the determinant for haemoglobin decrease.

In our work the characterization of the “kinetic” of serum B12 decrease overtime, in relation to the time elapsed from treatment, is relevant to evaluate the individual consumption of cobalamin and to consider when B12 should be retested in order to be accounted as a reliable index of B12 repletion and status. Accordingly, B12 retesting may be considered 3 months after the first dose of intramuscular hydroxocobalamin (1000 mcg) to evaluate the body pool. Anyway this preliminary data should be confirmed by additional studies specifically designed to evaluate the longitudinal trends of B12 levels after supplementation and their likely dependence on the baseline value. Further robust evidence are required to clarify how the kinetic of serum B12 may be of aid to avoid overtreatment and/or identify the population of newborns requiring a second dose and when to perform it. On the other hand, in this study the second administration is made *per* clinical protocol considering the available data in literature (a general consensus is lacking) [21,22] and the severe depletion at baseline more than value measured after administering the first dose. It is well known that one single dose triggers a variably high increase of B12 circulating levels, but this does not warrant the repletion of the body pool and overall the maintenance of the body pool overtime [1,21,22].

The evaluation of folate levels in the couple of mothers and newborns indicated that there is highest attention and compliance to folate supplementation since newborns at baseline show exceptionally

high levels and mothers show levels slightly below or around the recommended LRL for pregnancy/lactation, likely due to the physiological consumption occurring in pregnancy/lactation [23]. This is a first crucial issue, since both folate and B12 are involved as cofactors in various biological process that are critical for normal fetal growth and cognitive development [23,24]. The current clinical practice guidelines (CPGs) endorse folic acid (FA) supplementation in pregnant women, since this has been directly associated to the reduction of several congenital anomalies (neural tube and heart defects, urinary tract anomalies, oral facial clefts, and limb defects) [23]. This strong recommendation on FA does not extend to B12 supplementation, although, more recently, some authors have emphasized the crucial relevance to optimize both folate and vitamin B12 circulating levels in pregnant women through vitamin supplementation according to an individualized approach [25]. It has been evidenced that the interaction between highest levels of serum folate and lowest levels of vitamin B12, being a common finding in pregnant women, is associated to an increased risk of gestational diabetes [25].

Furthermore, in our study we had evidence of a two-fold increased rate of pre-term newborns with respect to the estimates obtained from the annual screened population of newborns. This result confirms the evidence from some authors who have reported that low B12 levels are associated with the increased risks of pre-term birth and of low birth weight [26]. Both conditions together with Hcy increase are further associated to the risk of developing future cardiovascular events [27-29].

Our study reinforces the need to include the recommendation of B12 supplementation in the CPGs, also as cost-saving strategy for the Healthcare.

This is the first study estimating the impact of B12 deficiency in newborns on the Healthcare. The costs for diagnosing and managing B12 deficiency in one newborn currently amounts to ~3253.16 € for the RHS, increasing to ~ 3550.31 € in the preterm case, and to 325.44 € for the caregiver. It is important to note that these costs are expected to grow according to the increase of cases with B12 deficiency, despite the declining rate of newborns/year, recorded in our Region as well as in Italy and all over the world [30]. Thus the increase of costs we have reported may be due to the intersection of two phenomena if we consider that in this case series a minority of subjects are Caucasian (42%). The first one is the fertility rate which is less affected in the non white ethnicities prevalent in our case series (Asiatic, Latinos and Africans) than in Caucasian ethnicity [31]. The second one is that the eating habits of the prevalent ethnicities with a preserved fertility rate (Asiatic) might increase the risk of B12 depletion in the newborns [22, 32,33]. Thus in the future we should expect higher proportion of newborns with B12 deficiency with respect to the past years.

From the RHS perspective, on average the costs for diagnosing and managing all newborns with B12 deficiency will change from ~188480 € /year to ~389089 €/year, within the next 5 years. From the caregiver perspective, the same costs will increase from ~19255 € /year to ~39714 € /year.

The management of B12 in newborns imposes a considerable burden on caregivers, not only in terms of financial costs but also in terms of stress and logistical commitment. For these reasons, there is a risk that an increasing number of families may not comply with the management, adversely affecting the population's health outcomes. Conversely, there would likely be good compliance among pregnant women for B12 supplementation, by following the example of FA supplementation. A recent study [34] performed on newborns reported seven cases of acquired B12 deficiency, describing biochemical parameters associated to cobalamin metabolism. Our study, is the first investigation performed on a large cohort, reporting clinical data from diagnosis to follow up and a description of maternal eating habits. A limitation of this study is the lack of a control group, which could enable us to explore the association of B12 levels with ethnicity and maternal eating habits to support the relevance of early nutritional care of the mother from conception onwards, and to promote efficient clinical identification and management of B12 deficiency during pregnancy.

Additional limitations in this study should be accounted, and in particular the use of different B12 methods and of decision thresholds to confirm the deficiency. It is well reported that the harmonization of B12 methods is poor, but the heterogeneity of the results reflects the management of outpatients in the routine clinical practice. On the other hand, the troublesome interpretation of results obtained from different assays on outpatients has been yet critically appraised [1,35]. An additional issue is the heterogeneity of the interpretative criteria shown in the different laboratory reports: reference ranges, risk thresholds, LRLs. Some statistical models have derived B12 LRL for characterizing vitamin deficiency based on the inverse and biphasic relationship between plasma concentrations of vitamin and functional indicators (i.e. Hcy, MMA). Most methods report a value of ~200 ng/L (relationship with MMA) others suggest a value of ~400 ng/L to increase the diagnostic sensitivity of the test (relationship with Hcy) [1,36]. As aforementioned, any effort to optimize the LRL for serum B12 might be impaired by the biological behaviour of the marker: the CV_w results so low as to imply a low ratio between CV_w and CV_g [37]. In this case, only monitoring serial individual changes is effective to classify patients, whereas a dichotomized transversal interpretation of a single result, according to a defined threshold, can be misleading [1]. The greatest majority of our patients was tested at baseline by assays measuring total B12 (i.e. including cobalamin bound both to transcobalamin and haptocorrin). In our case series we have discarded data from patients having a baseline B12 level measured by with holoTC (known as bioavailable fraction, and representing 5 –

20% of total vitamin) measurement which may provide a “pure” marker of vitamin absorption. The possibility to directly measure the B12 fraction potentially entering the cells by using holoTC assays might allow an early detection of impaired mechanisms of intake and may be endorsed in the case of newborns. Indeed, human milk contains high concentrations of unsaturated haptocorrin, which influence accurate measurement of cobalamins by using total B12 assays on serum samples, giving rise to falsely elevated or decreased results [38,39].

In conclusion, the severe B12 deficiency identified by NBS in newborns is not a negligible health issue, by considering the effect of B12 on growth and neurodevelopment and the costs for the healthcare system and the caregiver. Accordingly, preventing B12 depletion in newborns lowers healthcare costs and likely improves their health outcomes. The rate of newborns lost at follow up visit further reinforces the need to characterize and manage the deficiency during pregnancy. The incidence of cases collected in two years of clinical routine highlights that CPGs should endorse B12 supplementation and serial B12 testing during pregnancy, as it has been recommended for folate, to maintain a substantial body pool. A recent study underscores the relevance to identify and treat B12 low and marginal status during pregnancy [40]. Further studies are however required to address the clinical pathway to identify, treat and monitor by B12 serum testing, pregnant women with marginal and low B12 status, to assure a substantial B12 body pool also during fetal growth and neurodevelopment [41,42]. This could also prevent from an excess of the vitamin in the mother and newborn. A B12 overload could erroneously increase the levels of cobalamin related metabolism biomarkers detected by NBS and preclude the identification of methylmalonic acidemia, propionic acidemia or remethylation defects.

The prevention of B12 depletion in newborns further avoids to submit infants to serial blood draws (at least 4 in the first 3 months), to detect B12 and additional biomarkers. Currently several recommendations address to find solutions to lower the blood draws in newborns since blood tests is perhaps the most common source of painful procedure [43].

Acknowledgements: We thank the Regional Coordination Center for Laboratory Medicine of Lombardy for sharing the information from External Quality Assurance Services (EQAS) programs on the analytical performances of B12 and Hcy methods used in the laboratories of Lombardy.

Research funding: none to declare

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest

References

1. Ferraro S, Mozzi R, Panteghini M. Tracing a roadmap for vitamin B12 testing using the health technology assessment approach. *Clin Chem Lab Med* 2014;52:767-77.
2. Ferraro S, Verduci E, Zuccotti G, Sertori C. Reverse cascade testing from newborn screening: the opportunity to improve family healthcare outcomes. *Clin Chem Lab Med*. 2023; 62:e16-e18.
3. Kennedy D. B Vitamins and the Brain: Mechanisms, Dose and Efficacy—A Review. *Nutrients*. 2016; 8:68.
4. Temova Rakuša Ž, Roškar R, Hickey N, Geremia S. Vitamin B12 in Foods, Food Supplements, and Medicines-A Review of Its Role and Properties with a Focus on Its Stability. *Molecules*. 2022 ;28:240.
5. <https://sinu.it/larn/>(Accessed 19th June 2024)
6. Seetharam B, Alpers DH. Absorption and Transport of Cobalamin (Vitamin B 12). *Annu Rev Nutr*. 1982;2:343-369.
7. Kräutler B. Biochemistry of B12-Cofactors in Human Metabolism. In: Stanger O, ed. *Water Soluble Vitamins*. Vol 56. Subcellular Biochemistry. Springer Netherlands; 2012:323-346.
8. Rinaldo P., Zafari S, Tortorelli S and Matern D. Making the case for objective performance metrics in newborn screening by tandem mass spectrometry. *Ment Retard Dev Disabil Res Rev*. 2006;12:255-61.
9. Hall PL, Marquardt G, McHugh DM, Currier RJ, Tang H, Stoway SD, Rinaldo P. Postanalytical tools improve performance of newborn screening by tandem mass spectrometry. *Genet Med*. 2014;16:889-95.
10. https://esploradati.istat.it/databrowser/#/it/dw/categories/IT1,Z0500LAB,1.0/LAB_EMPLWAGE/DCSC_RACLI/IT1,533_957_DF_DCSC_RACLI_6,1.0 . Gross hourly earnings per hour paid for employee positions in euros (median). (Accessed 19th June 2024)
11. Revenue Agency. National tables of kilometer operating costs of cars and motorcycles developed by the ACI (2024). <https://www.informazionefiscale.it/IMG/pdf/23a0684000000010110001.pdf>. (Accessed 19th June 2024).

12. https://esploradati.istat.it/databrowser/#/it/dw/categories/IT1_POP_1.0/POP_BIRTHFERT/DCIS_NATI2/IT1_25_74_DF_DCIS_NATI2_1.1.0 (Accessed 19th June 2024).
13. Allen M, Poggiali D, Whitaker K, Marshall TR, van Langen J, Kievit RA. Raincloud plots: a multi-platform tool for robust data visualization. *Wellcome Open Res.* 2021 ;4:63.
14. Kaplan SS, Basford RE. Effect of vitamin B12 and folic acid deficiencies on neutrophil function. *Blood.* 1976;47:801-5.
15. Irevall T., Axelsson I., Naumburg E. B12 deficiency is common in infants and is accompanied by serious neurological symptoms. *Acta Paediatr.* 2017;106:101–104.
16. Ljungblad U.W., Astrup H., Mørkrid L., Hager H.B., Lindberg M., Eklund E.A., Bjørke-Monsen A.-L., Rootwelt T., Tangeraas T. Breastfed Infants With Spells, Tremor, or Irritability: Rule Out Vitamin B12 Deficiency. *Pediatr. Neurol.* 2022;131:4–12.
17. Jain R., Singh A., Mittal M., Talukdar B. Vitamin B12 deficiency in children: A treatable cause of neurodevelopmental delay. *J. Child Neurol.* 2015;30:641–643.
18. Ferraro S, Panteghini M. Folate and vitamin B12 assays after recalibration to the WHO International Standard 03/178: making the interpretation as simple as possible, but not simpler. *Clin Chem Lab Med* 2019;57:1112-1114.
19. Greibe E, Nexø E. Forms and Amounts of Vitamin B12 in Infant Formula: A Pilot Study. *PLoS One.* 2016;11:e0165458.
20. Jorgensen JM, Crespo-Bellido M, Dewey KG. Variation in hemoglobin across the life cycle and between males and females. *Ann N Y Acad Sci.* 2019 ;145:105-125.
21. Torsvik I, Ueland PM, Markestad T, Bjørke-Monsen AL. Cobalamin supplementation improves motor development and regurgitations in infants: results from a randomized intervention study. *Am J Clin Nutr.* 2013;98:1233-40
22. Goraya JS, Kaur S, Mehra B. Neurology of Nutritional Vitamin B12 Deficiency in Infants: Case Series From India and Literature Review. *J Child Neurol.* 2015;30:1831-7.
23. Ferraro S, Biganzoli G, Gringeri M , Radice S, Rizzuto AS , Carnovale C, Biganzoli EM, Clementi E. Managing folate deficiency implies filling the gap between laboratory and clinical assessment. *Clin Nutr* 2022;41:374-383.

24. Zou R, el Marroun H, Cecil C, Jaddoe VWV, Hillegers M, Tiemeier H, et al. Maternal folate levels during pregnancy and offspring brain development in late childhood. *Clin Nutr* 2021;40:3391e400.
25. Saravanan P, Sukumar N, Adaikalakoteswari A, Goljan I, Venkataraman H, Gopinath A, et al. Association of maternal vitamin B12 and folate levels in early pregnancy with gestational diabetes: a prospective UK cohort study (PRiDE study). *Diabetologia* 2021;64:2170–82.
26. Rogne T, Tielemans MJ, Chong MF, Yajnik CS, Krishnaveni GV, Poston L, et al. Associations of Maternal Vitamin B12 Concentration in Pregnancy With the Risks of Preterm Birth and Low Birth Weight: A Systematic Review and Meta-Analysis of Individual Participant Data. *Am J Epidemiol*. 2017;185:212-223.
27. Mercurio G, Bassareo PP, Flore G, Fanos V, Dentamaro I, Scicchitano P, Laforgia N, Ciccone MM. Prematurity and low weight at birth as new conditions predisposing to an increased cardiovascular risk. *Eur J Prev Cardiol*. 2013;20:357-67.
28. Bjørke Monsen AL, Ueland PM. Homocysteine and methylmalonic acid in diagnosis and risk assessment from infancy to adolescence. *Am J Clin Nutr*. 2003;78:7-21.
29. Ferraro S, Benedetti S, Mannarino S, Marcovina S, Mario Biganzoli E, Zuccotti G. Prediction of atherosclerotic cardiovascular risk in early childhood. *Clin Chim Acta*. 2024;552:117684.
30. Nargund G. Declining birth rate in Developed Countries: A radical policy re-think is required. *Facts Views Vis Obgyn*. 2009;1:191-3.
31. <https://www.pewresearch.org/social-trends/2012/05/17/explaining-why-minority-births-now-outnumber-white-births/> (Accessed 19th June 2024).
32. Gupta AK, Damji A, Uppaluri A. Vitamin B12 deficiency. Prevalence among South Asians at a Toronto clinic. *Can Fam Physician*. 2004;50:743-7;
33. Jeruszka-Bielak M, Isman C, Schroder TH, Li W, Green TJ, Lamers Y. South Asian Ethnicity Is Related to the Highest Risk of Vitamin B12 Deficiency in Pregnant Canadian Women. *Nutrients*. 2017 23;9:317.]
34. Scolamiero E, Villani GR, Ingenito L, Pecce R, Albano L, Caterino M, di Girolamo MG, Di Stefano C, Franzese I, Gallo G, Ruoppolo M. Maternal vitamin B12 deficiency detected in expanded newborn screening. *Clin Biochem*. 2014;47:312-7.

35. Ferraro S, Biganzoli G, Calcaterra V, Zuccotti G, Biganzoli EM, Plebani M. The relevance of establishing method-dependent decision thresholds of serum folate in pregnancy and lactation: when the laboratory stewardship meets the health-care needs. *Clin Chem Lab Med*, 2022; 60:1493.
36. Bjørke Monsen A.L., Ueland P.M. Homocysteine and methylmalonic acid in diagnosis and risk assessment from infancy to adolescence. *Am. J. Clin. Nutr.* 2003;78:7–21.
37. Harris EK. Effects of intra- and inter-individual variation on the appropriate use of normal range. *Clin Chem* 1974;20:1535 – 42.
38. Greibe E, Lildballe DL, Streym S, Vestergaard P, Rejnmark L, Mosekilde L, Nexø. Cobalamin and haptocorrin in human milk and cobalamin-related variables in mother and child: a 9-mo longitudinal study. *Am. J. Clin. Nutr.* 2013; 98: 389–395.
39. Lildballe DL, Hardlei TF, Allen LH, Nexø E. High concentrations of haptocorrin interfere with routine measurement of cobalamins in human serum and milk. A problem and its solution. *Clin Chem Lab Med* 2009; 47:182–7.
40. Chandyo RK, Kvestad I, Ulak M, Ranjitkar S, Hysing M, Shrestha M, et al. The effect of vitamin B12 supplementation during pregnancy on infant growth and development in Nepal: a community-based, double-blind, randomised, placebo-controlled trial. *Lancet*. 2023;401:1508-17.
41. Dror DK, Allen LH. Effect of vitamin B12 deficiency on neurodevelopment in infants: current knowledge and possible mechanisms. *Nutr Rev.* 2008 ;66:250-5.
42. Taskesen M, Yaramis A, Pirinccioglu AG, Ekici F. Cranial magnetic resonance imaging findings of nutritional vitamin B12 deficiency in 15 hypotonic infants. *Eur J Paediatr Neurol*. 2012;16:266-70.
43. Narvey M, Khashu M. It is high time we reduce "routine" blood work in neonatal units. *Front Pediatr*. 2023 ;11:1147512.

Table 1. Median concentrations (IQR) of biomarkers related to cobalamin metabolism measured at NBS on the first and second DBS.

	First DBS N = 87	Second DBS N = 87
Hours from birth	50 (49, 58)	312 (220, 357)
Metionine	15.6 (12.3, 18.9)	18.2 (15.1, 22.1)
Carnitine free (C0)	24 (17, 32)	26 (20, 30)
Acetylcarnitine (C2)	25 (19, 33)	11 (9, 15)
Propionylcarnitine (C3)	4.83 (3.77, 5.81)	1.90 (1.48, 2.66)
Butyrylcarnitine (C4)	0.27 (0.21, 0.39)	0.19 (0.16, 0.25)
Isovalerylcarnitine (C5)	0.12 (0.10, 0.16)	0.16 (0.13, 0.20)
Palmitoylcarnitine(C16)	3.98 (3.31, 4.99)	1.51 (1.00, 2.51)
3-OH-Palmitoylcarnitine (C16:OH)	0.050 (0.040, 0.060)	0.040 (0.030, 0.060)
MMA*	1.44 (0.67, 3.01)	2.82 (1.36, 3.88)
Hcy*	6.72 (5.35, 8.56)	5.76 (4.22, 7.98)
Ratios		
C3/C16	1.13 (0.91, 1.41)	1.12 (0.91, 1.74)
C3/Met	0.30 (0.23, 0.37)	0.10 (0.08, 0.15)
C4/C3	0.06 (0.04, 0.09)	0.10 (0.08, 0.13)
C5/C3	0.03 (0.02, 0.04)	0.08 (0.06, 0.11)

*MMA and Hcy measured as reflex test and thus unavailable for some samples.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

MMA was not detected in 21 cases (24.1%) on the first DBS and 60 cases (68.9%) on the second DBS. Hcy was not detected in 22 cases (24.0%) on the first DBS and 60 cases (68.9%) on the second DBS.

For Review Only

Table 2. Changes of biomarkers levels measured pre- and post- treatment

Markers of B12 deficiency	Pre-treatment	Post-treatment	Reference ^Range/Level	Units	Pre and post treatment difference
B12^^	209.0 (139.0-314.5)	1085 (831-1420)	200	ng/L	p < 0.001
pC3	1.05 (0.81-1.3)	0.42 (0.32-05)	>1.77	μmol/L	p < 0.001
Hcy^^^	13.2 (9.6-18.3)	3.5 (3.0-4.0)	>6.5	μmol/L	p < 0.001
pMMA	2.6 (1.3-4.0)	0.34 (0.19-0.54)	>4.0	μmol/L	p < 0.001
uMMA	29.9 (16.7-62.6)	3.55(2.78-5.08)	>2	mM/M	p < 0.001
Haemoglobin	12 (10.2-14.2)	10.7(10.1-11.8)	9-14	g/dl	p < 0.001
MCV	90.8 (87.5-94.0)	84(80.3-88)	77-115	fL	p < 0.001
neutrophils	1740 (1312.5-2530)	1690 (1235-2560)	1910-6230	10 ⁶ x L	p = 0.652

^ declared according to the used assay

^^ ECLIA Roche Cobas

^^^ CMIA Abbott Alinity

Legend to the Figures

Figure 1: Pie chart of mothers partitioned for ethnicity and diet regimens. B12 median levels within each partition (diet regimen and ethnicity).

Figure 2: Comparative visualization of biomarker levels detected pre- and post-treatment. Each plot displays the distribution and individual patient changes for: B12, C3, Hb, Hcy, MCV, pMMA, uMMA, and Neutrophils counts. The violin plots coupled with box plots show the distribution, while the connected points represent the individual trajectories. These trajectories are differently colored according to the time range elapsed (percentiles of the distribution) between pre- and post-treatment measurements.

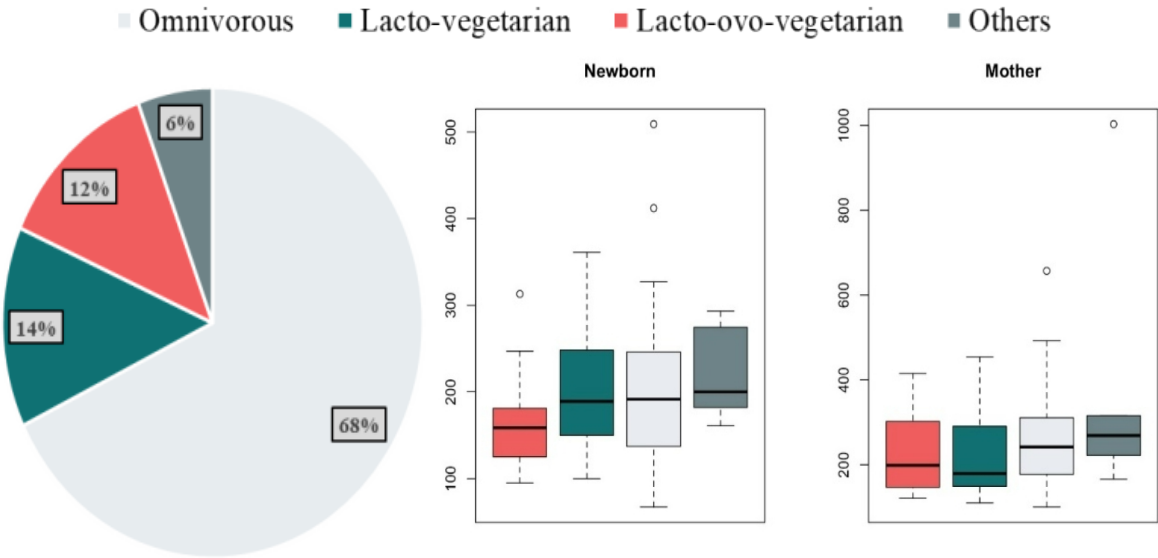
Figure 3: Scatterplot of B12 levels measured post-therapy (after the first dose) vs the time elapsed from treatment initiation. Residual magnitudes from fitted line are visualized, with color and size indicating the extent of the deviation. A LOESS smoothing line (in gray) provides a visualization of the kinetic pattern.

Figure 4: Total costs related to the identification and management of B12 deficiency in the newborns, from different perspectives (RHS and caregiver).

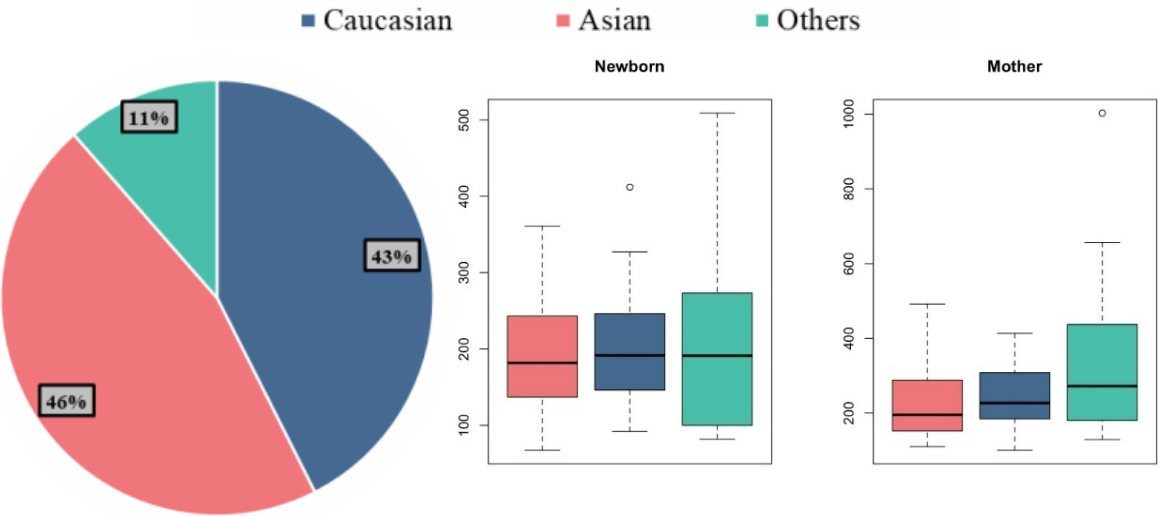
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Review Only

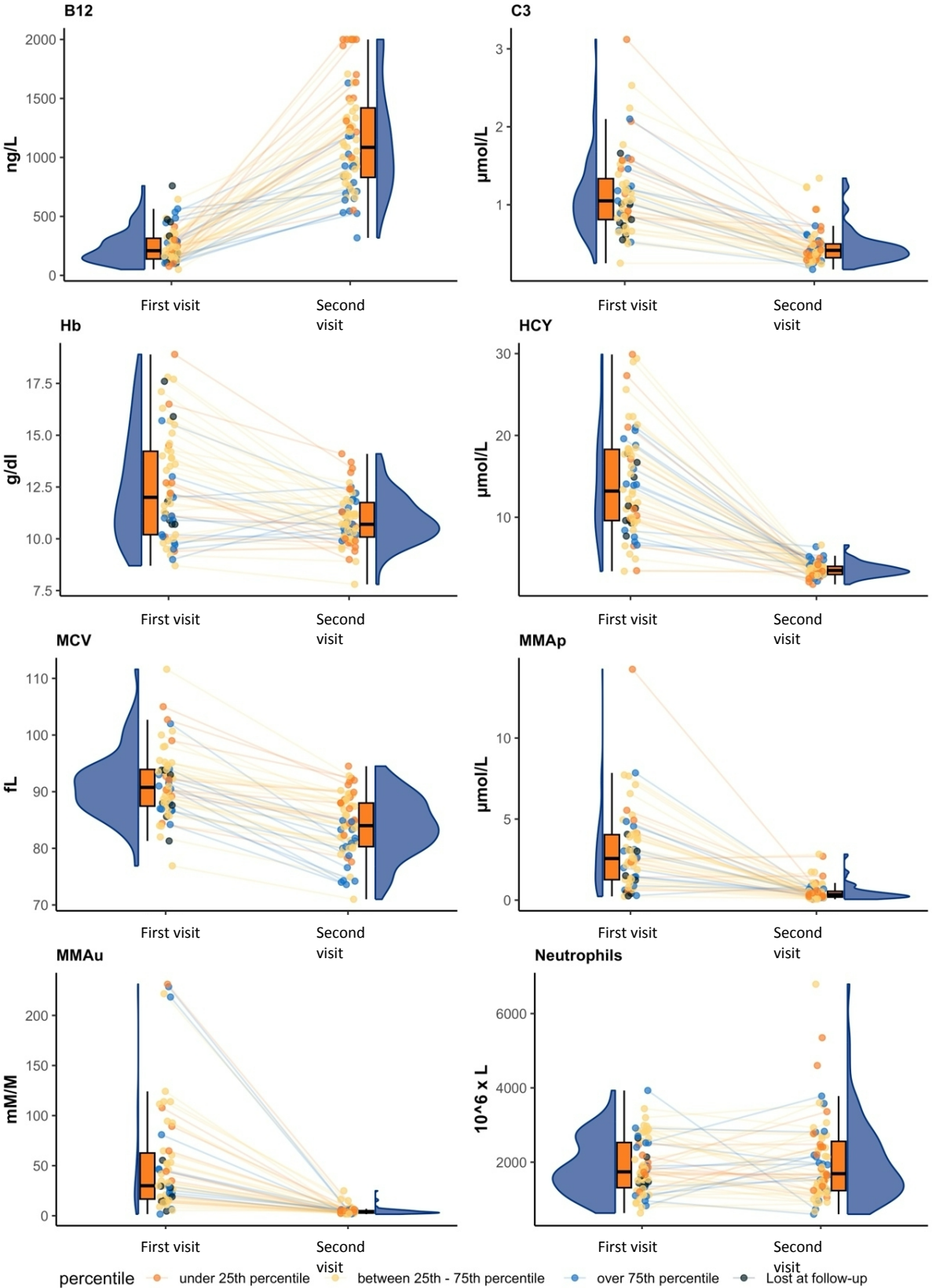
Diet type

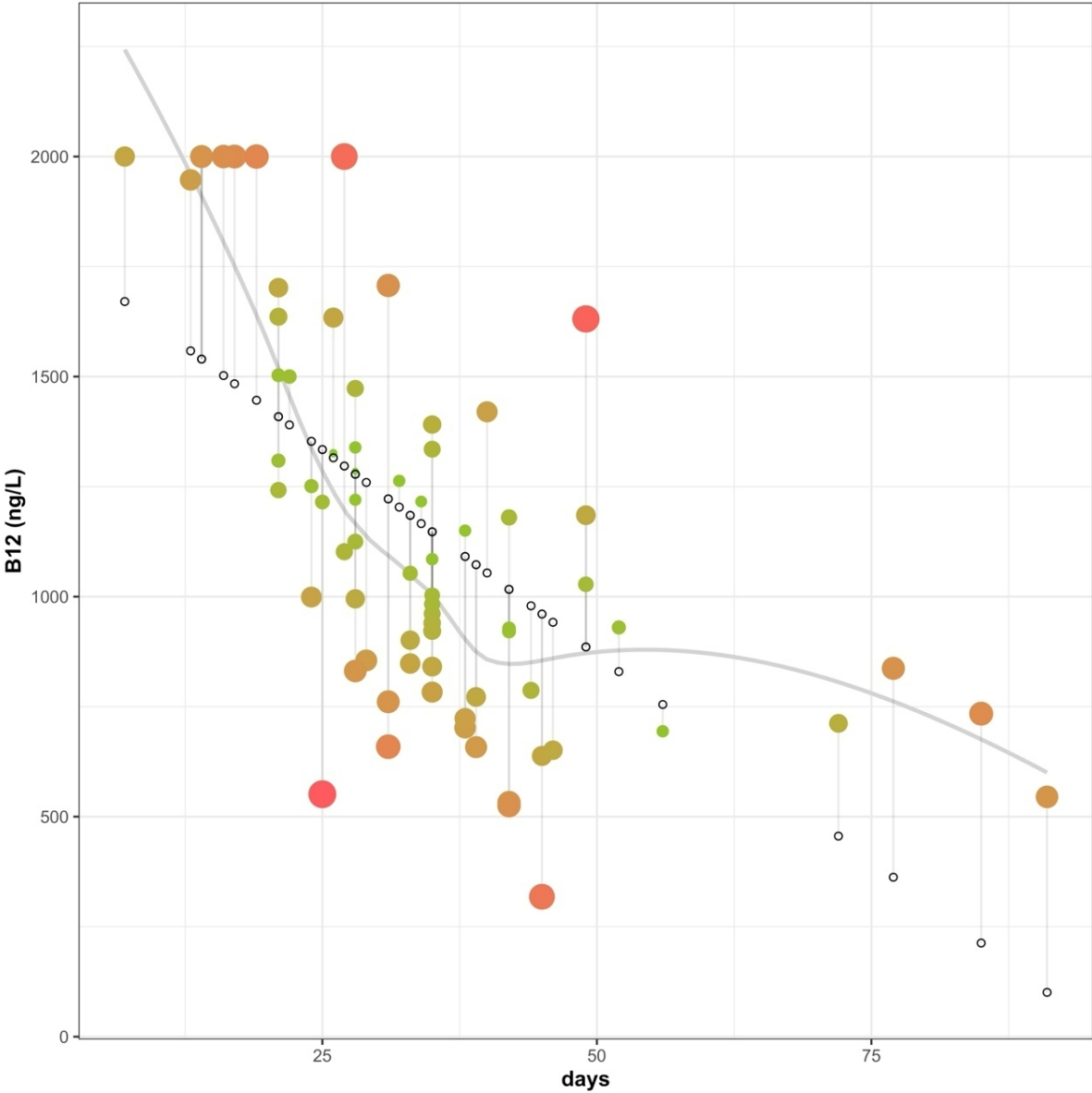


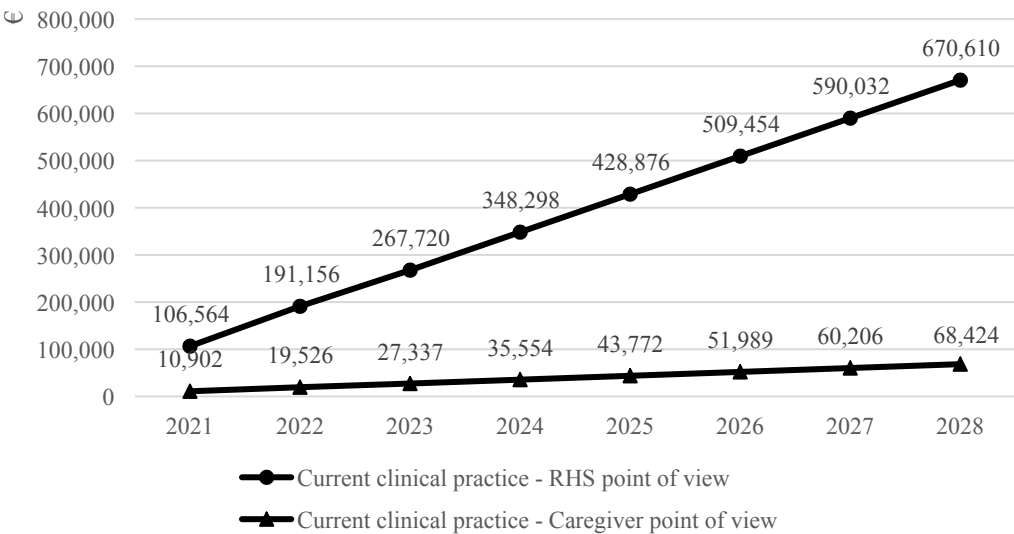
Ethnicity



Biomarkers post-treatment changes







Supplementary Table 1. Vitamin B12 Reference Intake levels of Nutrients and Energy for the Italian population and Dietary Reference Values (DRV) for the European Food Safety Authority (EFSA) during pregnancy and breastfeeding.

The Average Requirement (AR) refers to the level of nutrient intake that is sufficient to meet the requirements of 50% of healthy reference subjects.

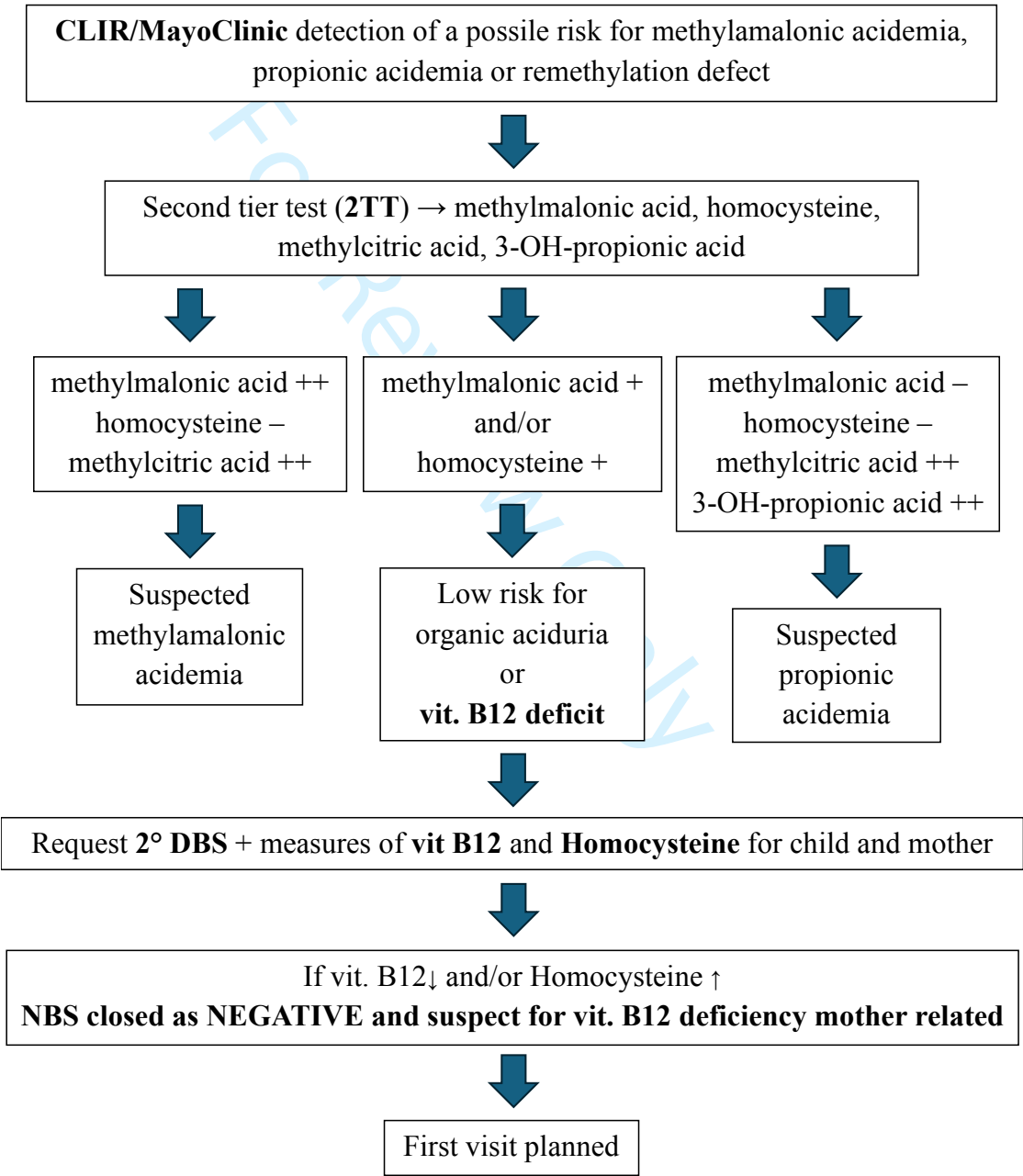
The Population Reference Intake (PRI) is sufficient to meet the requirements of 97.5% of healthy reference subjects.

The EFSA Dietary Reference Values are based on surveys in different European Union countries, with different average dietary intakes.

DRV of Nutrients and Energy for Italian population	Pregnancy	Breastfeeding
Average Requirement (AR)	2,2 µg/die	2,4 µg/die
Population Reference Intake (PRI)	2,6 µg/die	2,8 µg/die
EFSA DRV	Pregnancy	Breastfeeding
Adequate Intake (AI)	4,5 µg/die	5,0 µg/die

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Supplementary Figure 1. Flowchart describing the NBS biomarkers tested in newborns at possible risk of cobalamin metabolism defects according to Collaborative Laboratory Integrated Reports (CLIR – Mayo Clinic)[References 8-9].



Supplementary Table 2. Sources of information for cost analysis.

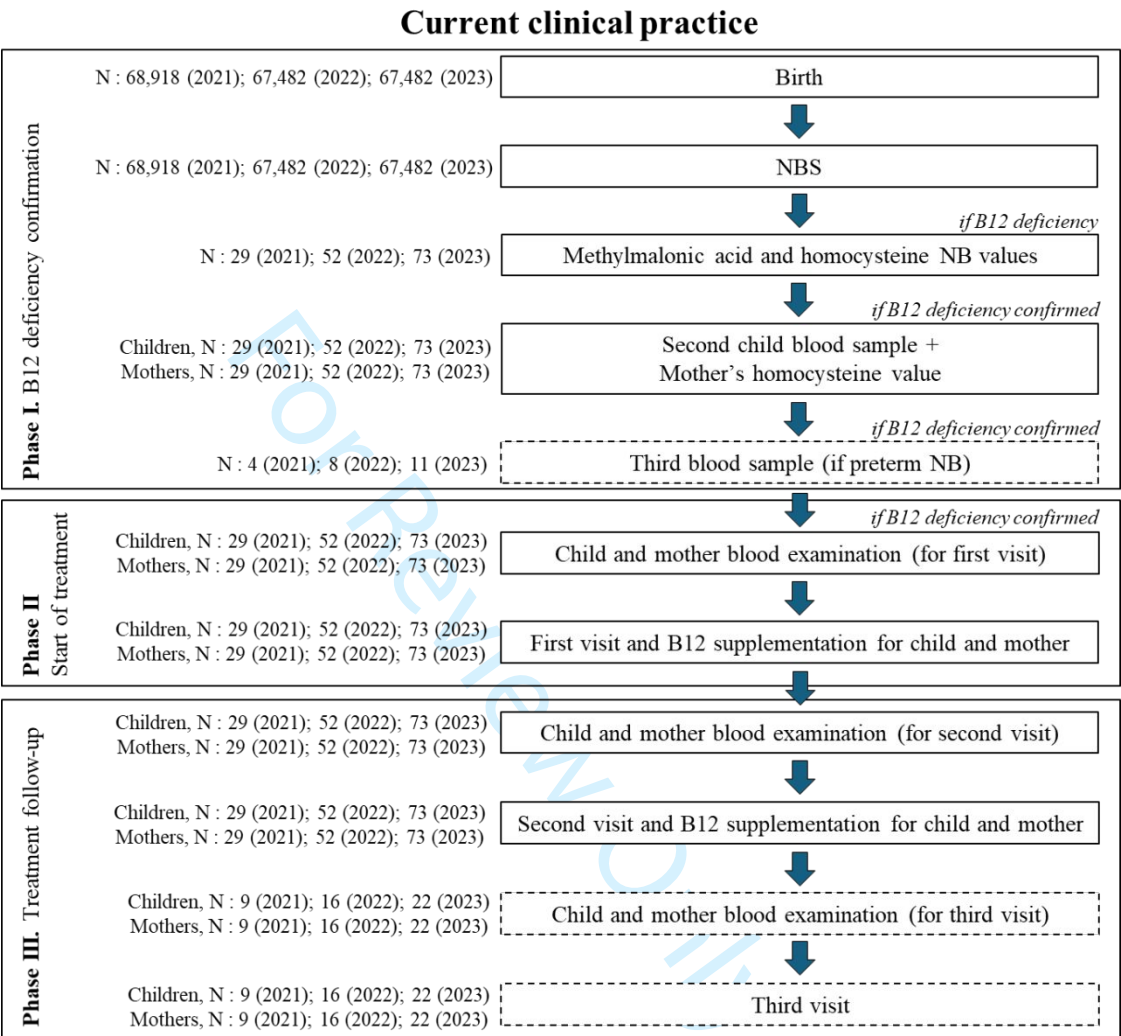
Perspective	Type of costs	Data	Source of information
RHS	Direct costs	RHS services costs	Reimbursement established by the RHS - Regional price list for laboratory/clinical test costs
	Cost-opportunity	<i>Cost of time</i> as: Healthcare professionals average hourly wage × Clinical activities time	Buzzi Children Hospital management control systems
			Clinicians interviews
Caregiver	Direct costs	<i>Caregivers' travel costs</i> as: Average distance travelled × Cost per km	Buzzi Children Hospital data – Patients birth points table Automobile club of Italy table 2024 [Reference 11]
	Cost-opportunity	<i>Cost of time</i> as: Caregivers' average hourly wage × Caregivers' time for travel and clinical activities	Italy's National Institute of Statistics [Reference 10] Buzzi Children Hospital management control systems and clinician's interview

* The total number of newborns included is retrieved from Italy's National Institute of Statistics [Reference 12].

** The number of newborns with B12 deficiency is retrieved from Regional Reference Laboratory for Neonatal Screening (RRLNS), and Metabolic Diseases and Clinical Nutrition Service of the Buzzi Children Hospital

Supplementary Figure 2.

Clinical pathway for diagnosis and management of B12 deficiency partitioned into 3 phases: B12 deficiency confirmation, start of treatment and follow-up. The actual sample size of newborns with B12 deficiency for each year is reported.



The diagnosis and management B12 deficiency in newborns may be partitioned in three phases, to allow an accurate estimation of the costs.

Phase I includes the samples and the laboratory tests required to confirm B12 deficiency, in newborns and their mothers, considering that in cases of preterm birth generally one additional DBS is required.

Phase II encompasses the start of treatment, which includes clinical evaluation, laboratory tests, specialist consultation with dietary and clinical nutrition evaluation, and neurological assessment. During the first visit, the newborn receives intramuscular B12 supplementation. The same laboratory tests are required for the mother, with

recommendations for intramuscular B12 supplementation and folic acid supplementation until the subsequent check-up.

Phase III involves treatment follow-up, repeating the procedures of Phase II.

Supplementary Table 3. RHS and caregiver costs (single case).

	RHS		Caregiver		Total (€)
	Direct costs (€)	Cost-opportunity (€)	Direct costs (€)	Cost-opportunity (€)	
Phase I					
<i>Phase I</i>	520	16.63	/	/	536.63
<i>Premature newborn</i>	808	25.78			833.78
Phase II	1388.34	98.26	63.76	98.96	1649.32
Phase III	1131.67	98.26	63.76	98.96	1392.65

Supplementary Table 4. Cut-off of the biomarkers reported in table 1 adjusted for age and weight

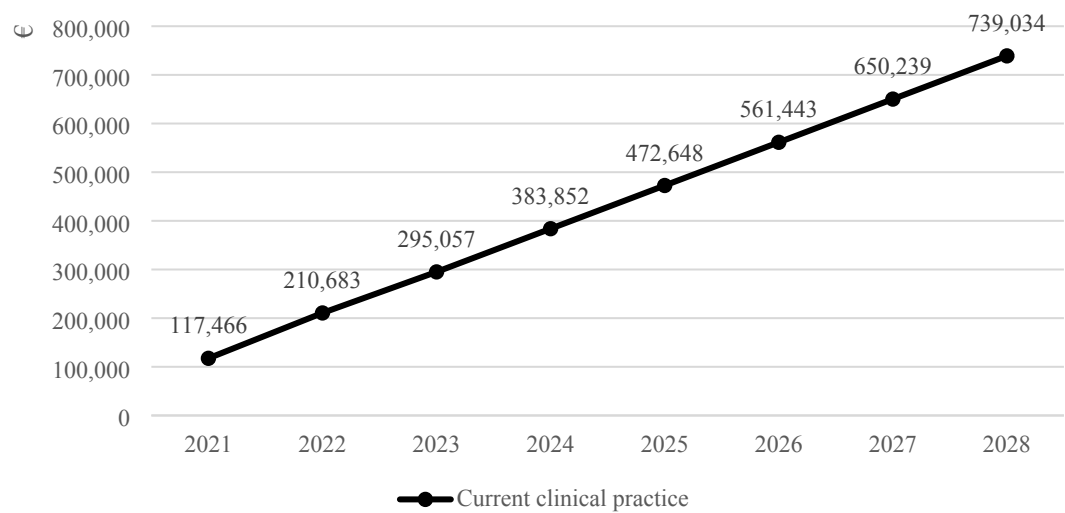
Age	<1500g low-high	1500g<x<2000g low-high	2000g<x<2500g low-high	>2500g low-high	Units
48-72 h					
MET	10,33-91,15	8,45-62,89	9-44,96	9,14-36,04	μmol/L
C0	10,49-101,39	9,98-86,12	8,64-61,14	7,09-45,98	μmol/L
C2	9,14-63,52	8,53-63,04	9,11-58,02	10,17-56,11	μmol/L
C3	1,08-7,45	0,97-7,07	0,95-6,05	0,95-5,43	μmol/L
C4	0,14-1,42	0,11-0,96	0,09-0,82	0,09-0,86	μmol/L
C5	0,1-0,99	0,07-0,61	0,06-0,37	0,05-0,29	μmol/L
C16	0,46-4,57	0,85-6,29	1,18-7,07	1,68-7,46	μmol/L
C16:OH	0,02-0,09	0,02-0,1	0,02-0,09	0,02-0,09	μmol/L
MMA	2,5	2,5	2,5	2,5	μmol/L
HCY	6,5	6,5	6,5	6,5	μmol/L
C3/C16	0,49-6,13	0,32-4,29	0,26-2,39	0,25-1,54	
C3/MET	0,03-0,54	0,03-0,47	0,04-0,40	0,05-0,41	
C4/C3	0,06-0,51	0,05-0,37	0,04-0,39	0,04-0,36	
C5/C3	0,04-0,36	0,03-0,24	0,03-0,17	0,02-0,13	
336-384 h					
MET	10,19-59,72	9,01-49,28	10,98-48,61	11,7-44,64	μmol/L
C0	4,76-105,35	8,29-74,71	10,75-47,13	11,01-45,23	μmol/L
C2	2,38-51,96	3,7-29,96	4,28-20,4	4,26-18,91	μmol/L
C3	0,22-4,11	0,28-2,59	0,31-1,75	0,33-1,98	μmol/L
C4	0,08-0,78	0,08-0,45	0,07-0,38	0,07-0,37	μmol/L
C5	0,07-0,93	0,08-0,48	0,08-0,37	0,07-0,36	μmol/L
C16	0,27-3	0,35-2,67	0,3-2,19	0,32-2,29	μmol/L
C16:OH	0,01-0,08	0,01-0,08	0,01-0,07	0,01-0,08	μmol/L
MMA	2,5	2,5	2,5	2,5	μmol/L
HCY	6,5	6,5	6,5	6,5	μmol/L
C3/C16	0,2-3,59	0,21-2,77	0,27-2,5	0,26-2,41	
C3/MET	0,01-0,17	0,01-0,12	0,01-0,11	0,01-0,11	
C4/C3	0,1-0,75	0,1-0,63	0,09-0,65	0,09-0,57	
C5/C3	0,11-0,75	0,11-0,68	0,1-0,57	0,08-0,51	

Supplementary Table 5. Estimates of the coefficients of the multivariate model investigating the relationship between B12 levels and C3, Met, C16:OH (in bold) adjusting for type of diet, ethnicity and gestational age. A and B refer to the first and the second DBS.

A			
	coefficients	lower bd	upper bd
Intercept	0.78482	0.35597	1.89074
C3	-0.04009	-0.08225	0.02736
MET	0.01497	-0.00343	0.03522
C16:OH	-2.83609	-6.01829	3.66488
Caucasian	-0.16903	-0.34242	0.08612
Ethnicity (Other)	0.25445	-0.51329	0.47961
Lacto-Vegetarian	0.16153	-0.06078	0.39230
Omnivore	0.20536	0.05450	0.42772
Other diets	0.21805	-0.15898	0.44897
Preterm	0.24781	-0.19639	0.49936
Non adequate Intake	0.17612	-0.14089	0.25201
B			
Coefficients:			
	coefficients	lower bd	upper bd
Intercept	0.83267	0.49244	1.31285
C3	0.00547	-0.05557	0.09690
MET	0.00285	-0.01310	0.01280
C16:OH	-3.67834	-6.99710	2.82195
Caucasian	-0.10988	-0.29657	0.10046
Ethnicity (Other)	0.29626	-0.63173	0.57602
Lacto-Vegetarian	0.14966	-0.06702	0.35627
Omnivore	0.15178	-0.01195	0.32463
Other diets	0.10944	-0.39238	0.54877
Preterm	0.27192	0.00657	0.47311
Non adequate Intake	0.11699	-0.09871	0.25180

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Supplementary Figure 3. Projection of the total costs of diagnosis and management of B12 deficiency/year.



Supplementary Table 6. RHS and caregiver costs (all cases), for the current approach

	2021	2022	2023
Direct costs of RHS (€)	99,461	178,427	249,897
Costs-opp RHS (€)	7,102	12,729	17,822
Direct costs of caregiver (€)	4,272	7,651	10,712
Costs-opp caregiver (€)	6,630	11,875	16,625
Total (€)	117,466	210,683	295,057

For Review Only

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Review Only