Newborn screening for homocystinurias: recent recommendations versus current practice

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Abbreviations

C2	acetylcarnitine
C3	propionylcarnitine
C17	heptadecanoylcarnitine
CBSD	cystathionine beta-synthase deficiency
CLIR	Collaborative Laboratory Integrated Reports
cobalamin	cbl
cRMD	combined remethylation disorder
DBS	dried blood spots
iRMD	isolated remethylation disorder
MAT I/III D	methionine adenosyltransferase I/III deficiency
MMA	methylmalonic acid
Met	methionine
MoM	multiples of the median
MTHFRD	methylenetetrahydrofolate reductase deficiency
NBS	newborn screening
Phe	phenylalanine
RMD	remethylation defect
tHcy	total homocysteine

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ABSTRACT

Purpose: To estimate the performance and accordance with published recommendations of the current practice of newborn screening (NBS) for homocystinurias .

Methods: Twenty two of 32 NBS programs from 18 countries screened for at least one homocystinuria. Centers provided pseudonymized NBS data from patients with deficiency of cystathionine beta-synthase (CBSD, n=19), methionine adenosyltransferase I/III (MATI/IIID, n=28), combined remethylation defects (cRMD; n=56) and isolated RMD (iRMD) including methylenetetrahydrofolate reductase deficiency (MTHFRD) (n=8). Markers and decision limits were converted to multiples of the median (MoM) to allow comparison between centres.

Results: NBS algorithms and decision limits varied considerably. Only nine centres used the recommended second tier parameter total homocysteine (tHcy). Median decision limits of all centres were ≥2.35 for high and ≤0.44 MoM for low methionine; ≥1.95 for high and ≤0.47 MoM for low methionine/phenylalanine, ≥2.54 for high propionylcarnitine and ≥2.78 MoM for propionylcarnitine/acetylcarnitine. Use of these decision limits alone had 100%, 100%, 86%, and 84% sensitivity for detection of CBSD, MATI/IIID, iRMD and cRMD, respectively. However, even these decision limits missed six individuals with cRMD which had been not been detected by NBS. To enhance sensitivity and decrease second tier tHcy testing costs we further adapted these decision limits using data of 15,000 healthy newborns.

Conclusions: This study demonstrates that the practice of NBS for homocystinurias largely does not follow recently published recommendations. We propose the use of median MoM-corrected decision limits combined with second tier tHcy analysis to improve NBS for homocystinurias.

KEY WORDS: remethylation; cobalamin, methylenetetrahydrofolate reductase (MTHFR) deficiency, newborn screening.

Compliance with ethical guidelines

All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000. This study was part of the EHOD project and has ethical approval in Zürich (KEK-Switzerland Nr.2012-0020) and in local centres as required

Conflict of Interest

M Huemer has received research grants from Nutricia and SOBI and honorariums for lectures from Nutricia, Recordati Rare Disease Foundation, Shire and Genzyme. HJ Blom received a research grant from Orphan Europe. S Kölker receives financial support for the E-HOD registry management by the European Union, for the Cystadane Surveillance Protocol by Orphan Europe, and for a pilot newborn screening study including homocystinurias and methylation defects by the Dietmar Hopp Foundation, St. Leon-Rot, Germany. MR Baumgartner declares that the University Children's Hospital Zurich has received educational and research grants from Actelion, Genzyme and Milupa Metabolics and receives support for the E-HOD Registry/Cystadane surveillance program from Orphan Europe. AA Morris has received honoraria for lectures from Nutricia and Recordati Rare Disease Foundation. V Kožich declares that the Charles University-First Faculty of Medicine has received support from the Recordati Rare Disease Foundation for organizing an educational course on homocystinurias and methylation defects, and reimbursement for laboratory analyses from Orphan Technologies. C Dionisi-Vici has received research grants, speaker and consultancy honoraria from Nutricia, Medifood, SOBI and Dr. Schär Medical Nutrition. AM Lund and RH Zetterström have received grants and travel reimbursement from Orphan Europe, Nutricia and SOBI. E Crushell has received an honorarium for a lecture from Nutricia Metabolics. G la Marca has received travel reimbursement from Nutricia and research grants from Genzyme. C Pedron-Giner has received support from Vitafló-Nestlé España to attend SSIEM meetings. ~~hhonorariums for lectures from Nutricia and Mead Johnson and travel reimbursement from Nutricia, Vitaflo-Nestlé España and SOBI.~~ R Keller, P Chrastina , J Bartl, S Gouveia, A Ribes, F Gleich, M Pavlíková declare that they have no relevant conflict of interest.

Details of the contributions of individual authors

R Keller conducted and coordinated the study and drafted the manuscript. M Huemer and V Kožich provided the original concept of the study, coordinated the study and revised the manuscript. P Chrastina, M Pavlíková and J Bártl conducted the statistical analyses and drafted the respective parts of the manuscript. S Gouveia and A Ribes coordinated data collection from Spanish centres. F Gleich and S Kölker managed the E-HOD registry and provided pseudonymized data on behalf of the E-HOD consortium. MR Baumgartner, C Dionisi Vici, AA Morris, and HJ Blom critically revised the final draft of the manuscript. The members of the E-HOD consortium contributed and discussed data from their local NBS programmes. All authors critically revised and approved the final manuscript.

INTRODUCTION

Homocystinurias are rare genetic diseases caused by deficient activity of enzymes involved in the metabolism of sulfur amino acids or of the related B-vitamins. Although these diseases are etiologically and clinically heterogeneous they share the biochemical feature of elevated concentrations of homocyst(e)ine in blood and urine. This study focuses on classical homocystinuria or cystathionine beta-synthase deficiency (CBSD), methionine adenosyltransferase I/III deficiency (MAT I/IIID), the combined remethylation defects (cRMD), cblC, cblD-MMA-Hcy, cblF, cblJ, and the isolated RMD (iRMD) cblD-Hcy, cblE, cblG, and methylenetetrahydrofolate reductase (MTHFR) deficiency.

Clinical manifestation of untreated homocystinurias depends on the affected gene and the severity of mutations. Often cognitive impairment, seizures, white matter and ocular abnormalities, connective tissues involvement and thromboembolism are presenting signs. Details on clinical features, diagnostic and therapeutic strategies and efficacy of treatment were recently reviewed in this journal (Morris et al 2017, Huemer et al 2017). The evidence discussed in these and other publications (Yap & Naughten 1998; Yap 2012; Gan-Schreier et al 2010; Weisfeld-Adams et al 2010, Weisfeld-Adams et al 2013, Carrillo-Carrasco et al 2012, Martinelli et al 2011, Huemer et al 2014, Diekman et al 2014) indicates that patients with homocystinurias may benefit from early treatment. The opportunity window for efficient intervention seems to be quite narrow—especially in RMD—requiring timely diagnosis, preferably by newborn screening (NBS).

Ideally, NBS programs reliably, by using high quality, economically feasible screening tests, detect patients suffering from a well-characterised disorder in which early treatment is beneficial (Wilson & Jungner 1968). In the past decades, different NBS strategies for the homocystinurias have been developed and three recent publications congruently proposed to adopt two-tier screening strategies (Huemer et al 2015, Okun et al 2017, Gramer et al 2017). In the first step, the following primary markers should be assessed from dry blood spots (DBS): (a) elevated methionine (Met) and/or methionine-to-phenylalanine (Met/Phe) ratio for CBSD; (b) low Met and/or Met/Phe levels for RMD; and (c) elevated propionylcarnitine (C3), propionyl/acetylcarnitine (C3/C2) ratio and possibly heptadecanoylcarnitine (C17) for the cRMD (Huemer et al 2015, Malvagia et al 2015, Gramer et al 2017). The second tier markers total homocysteine (tHcy) for all homocystinurias and methylmalonic acid (MMA) for cRMD,

are subsequently analysed in the small number (~1%) of DBS with abnormal concentrations of primary markers.

Establishment of optimal decision thresholds is among the most challenging tasks for NBS programs. Decision limits closer to the median of the marker in the population of healthy newborns increase sensitivity but simultaneously increase the false positive rate which determines the cost of second-tier testing. While decision limits more distant from the population median reduce the false positive rate, they may fail to detect patients.

It has been suggested that NBS centres should pool data and share experience on the NBS algorithms for homocystinurias as proposed by Gramer et al 2017. However, the comparability of biomarker data among different NBS centres is a generic problem of population screening programs due to e.g. different analytical platforms, times of sampling, and decision thresholds. The use of Z-scores or multiples of median (MoM) has been proposed to facilitate pooling data across various screening programs (Burke et al 2016).

This work was part of the EU-funded "European network and registry for homocystinurias and methylation defects" (E-HOD; www.e-hod.org) project and investigates the current practice of NBS for homocystinurias in 18 countries involved in the E-HOD project. We analysed the spectrum of screened disorders, screening strategies and their conformity with recently published recommendations (Gramer et al 2017; Okun et al 2017; Huemer et al 2015). Furthermore, this study evaluated the variation in decision limits and examined whether the use of MoM-corrected data could improve the performance of NBS for the homocystinurias.

METHODS

Data acquisition

EHOD partners were invited to answer a survey addressing their regional practice of NBS for the homocystinurias with the following key questions:

a) Are homocystinurias part of your NBS panel?
b) Which algorithms do you use to detect homocystinurias?
c) Does your program use second tier testing for tHcy?
d) What are the median values of Met, Met/Phe ratio, C3 and C3/C2 ratio markers in the population of healthy newborns in your centre?
e) Which decision limits do you use to flag the result of NBS as abnormal?

Pseudonymised data sets from 141 patients (from 23 centres) with CBSD, MAT I/IIID, MTHFRD and cblC, cblD, cblJ, cblE and cblG defects were extracted from the EHOD registry; one additional E-HOD centre contributed data sets from ten patients not yet included into the registry (n=151). Only patients younger than 10 years at data entry were selected to facilitate retrieval of data on NBS markers from local sources.

Forty patients had to be excluded from further analyses due to incompleteness of data, resulting in a final sample of 111 individuals. Four groups of patients were defined: CBSD (n=19); MAT I/IIID (n=28), cRMD (cblC, cblD, cblJ) (n=56), and iRMD (MTHFR, cblE, cblG) (n=8). For CBSD, no subgroup classification according to pyridoxine responsiveness was attempted, as no precise information on the vitamin B6 response criteria in individual centres was available.

Calculation of MoM-corrected decision limits and marker values in patients

To allow comparison of data across populations and centres using different screening platforms we calculated MoM of the Met, Met/Phe, C3 and C3/C2 decision limits reported by the NBS centres by dividing values of each marker by its population median. Transfer of individual NBS marker values of patients with homocystinurias to MoM- was performed accordingly.

Performance of decision limits

To assess the performance of the highly dispersed decision limits we calculated the range and median decision limits for each MoM-normalized marker. In a subset of patients with complete data sets we evaluated sensitivity of the extreme values and of the median of decision limits by calculating the proportion of patients with homocystinurias that would be detected by the respective decision limit. In addition, we analysed the sensitivity of a combination of markers and created respective two-dimensional plots. The Kruskall-Wallis test for independent samples was used to compare NBS parameters between detected and non-detected individuals.

Modelling sensitivity, specificity and cost-efficacy in one NBS program

For this analysis, data on markers in 15,000 healthy newborns from one NBS program were combined with data of the 111 patients reported in this study. For each of the four targeted (groups of) disorders (CBSD, MAT I/IIID, iRMD and cRMD) we examined how the use of specific decision limits would influence the sensitivity and specificity of the combination of markers in the model population. Firstly, we constructed two-dimensional grids for Met and Met/Phe, or C3 and C3/C2 values for the combined pool of controls and patients. Next, we computed sensitivity and specificity of two scenarios, i.e. when both markers would be exceeding decision limits (marked as "AND" test) or when at least one of both markers would be crossing the decision limits (marked as "OR" test). To compare the sensitivity and specificity of these marker combinations we constructed receiver operator curves (ROC). Finally, we used the dataset to model specificity as an important determinant of either false positive rate or of the cost of second tier testing by constructing contour plots of sensitivity of markers with specificity fixed at 99% and above. The annual cost-savings/expenditure of the number of second tier tHcy tests was calculated from the price of tHcy analysis in DBS according to the reimbursement rate determined by the national health authority (approximately € 30.90 per analysis).

Statistical software

Computations and model testing were performed in the statistical language and environment R (v. 3.2.2.), and SPSS and NCSS statistical software, respectively.

RESULTS

NBS programmes for homocystinurias in surveyed countries

Thirty-two centres from 16 European and 2 non-European countries contributed data on their NBS panels operating in 2016. The number of primarily targeted disorders varied considerably among countries as well as among regional programmes within countries. Twenty-one centres screened for CBS deficiency and 15 primarily targeted cblC disease. The Italian, Czech and Spanish centres and Qatar (analyses for Qatar performed by the NBS Centre Heidelberg, Germany until 2016) also screened for iRMD (MTHFRD and/or isolated cobalamin-related remethylation defects). MAT I/IIID was a primary target in Austria, in Italian and some Spanish centres. Data from the 2011 survey in 16 European countries (Loeber et al 2012) and the present study cannot easily be compared due to different grouping of disorders and countries; however, four countries initiated screening for CBSD and two for cRMD as primary target in at least one centre since 2011 (Loeber et al 2012).

NBS algorithms

Detailed information on NBS algorithms was available for 19 centres reporting a variety of combinations and sequences of use of Met, Met/Phe, C3 and C3/C2 markers. tHcy was used as a primary marker only in Qatar. Although recent recommendations propose so, only 9 of 24 centres adopted a two tier-strategy with tHcy as second-tier marker. As an example of the complexity and heterogeneity of algorithms in different NBS programs supplementary figure 1 summarises the approaches targeting the cblc defect and other cRMD.

Decision limits for Met, Met/Phe, C3 and C3/C2

To test whether differences in decision limits among programs are caused by different concentrations or ratios of markers in the respective normal newborn populations we firstly compared the medians of each marker (see Figure 1 and Figure 2). Distribution of markers in healthy newborns varied substantially among centres. Medians for Met, Met/Phe, C3 and C3/C2 ranged from 12.8-23 µmol/L, 0.22-0.51, 1.27-2.1 µmol/L and 0.057-0.18, respectively. It is however of note that the dispersion of decision limits for Met, Met/Phe, C3 and C3/C2 was even wider than the dispersion of medians indicating a substantial lack of consensus on optimal thresholds to flag values as abnormal (Figure 1, Figure 2 and supplementary table 1).

Sensitivity of decision limits to detect patients with homocystinurias

In the subset of patients with complete data sets we evaluated sensitivity of decision limits.

Performance of centres' decision limits. The proportion of patients with homocystinurias that would be detected by the respective the centres' decision limits varied considerably and clearly showed that some programs use decision limits with unsatisfactory sensitivity (Supplementary Table 1).

Since there is no consensus on optimal decision thresholds, we examined in the next step sensitivity of the medians of decision limits of all programmes (as the possible best proxy of consensual values), as well as the sensitivity of combinations of parameters. These medians of MoM-normalized decision limits of all centres are labeled as "suggested cut-offs" in the following paragraphs.

Performance of median decision limits of high Met and Met/Phe to detect CBSD and MATI/IIID. The suggested cut-offs for high Met (≥2.35 MoM) or high Met/Phe (≥1.95 MoM) detected all 17 patients with CBSD and all 28 patients with MATI/IIID in this study. Congruently, the combination of these markers detected all patients (Supplementary table 1, Supplementary table 2, and Figure 1).

Performance of median decision limits of low Met and Met/Phe to detect iRMD. The study cohort contained data on Met from eight and Met/Phe from seven patients with iRMD. The suggested cut-off for low Met (≤0.44 MoM) detected only three of eight patients; low Met/Phe (≤0.47 MoM) would detect six of seven patients. The combination of low Met and/or low Met/Phe would detect six of seven patients (Supplementary table 1, Supplementary table 2, Supplementary table 3, Figure 2).

Performance of median decision limits of low Met and Met/Phe, and high C3 and C3/C2 to detect cRMD. The group of patients with cRMD with any information on decision limits consisted of 47 subjects, of which six had been missed by their original NBS program (another cRMD patient missed by NBS was excluded from these analyses due to missing information on decision limits). Data sets (all four markers) were not in all patients complete.

Sensitivity of the suggested cut-offs for low Met (≤0.44 MoM) and low Met/Phe (≤0.47 MoM) was poor, detecting only 22 and 28 of 47 patients, respectively. In contrast, the suggested cut-offs for high C3 (≥2.54 MoM) and C3/C2 (≥2.78 MoM) performed better detecting 41 of 47, and 33 of 45 patients, respectively. The combination of all four markers yielded slightly better

results. At least one marker was outside the reference range in 43 cases. In summary, the combination of all four markers detected more patients with cRMD compared to the use of only single markers but their performance was not sufficient to detect all patients.

In order to shed more light on the subgroup of the seven cRMD patients missed by their local NBS programs we conducted a detailed analysis of the NBS parameters obtained at the time of screening. These patients exhibited significantly higher MoM-normalized Met ($p=0.03$) and lower MoM-corrected C3 ($p=0.004$) and C3/C2 ($p=0.027$) compared to 40 individuals detected by regional NBS programs (Supplementary Table 4). These analyses demonstrate that especially cRMD patients with milder biochemical phenotype at the time of blood sampling may be missed by NBS even if consensual decision limits would be used.

Sensitivity, specificity and cost-efficacy in a model NBS program using median decision limits of all NBS programs

Analysis of specificity and sensitivity requires not only data on marker values in patients but also data on marker distributions in the population of healthy newborns. As an example how data from this study may be used for optimizing NBS programs we modelled these parameters using data on Met and Met/Phe in 15,000 newborns from one selected NBS program.

Left panels in Figure 3 show receiver operating characteristic curves for different markers and truth-functional operators (i.e. the AND combination of two markers or the OR combination of two markers). The area under the ROC curve is over 0.99 in CBSD using either high Met AND high Met/Phe, or high Met OR high Met/Phe. For cRMD the area under ROC curve was substantially higher for marker combinations employing the high C3 AND/OR high C3/C2 (0.97 and 0.98, respectively) compared to combination of low Met AND/OR low Met/Phe (0.84 and 0.87, respectively).

The middle and right panels of Figure 3 show nomograms of specificity at different fixed sensitivities and nomograms of sensitivity at fixed specificity of 0.99. In this study the nomograms were used to optimize the decision limits of a single NBS program for homocystinurias. Lowering of the present decision limits for high Met and high Met/Phe from 2.7 to 2.56, and 2.27 to 2.08, respectively, increased sensitivity for detecting CBSD from 0.94 to 1.0; the number of second-tier tests increased by only 0.03 % with a cost increase from € 4,942 to € 5,766 per year. For iRMD, an increase of the decision limits for low Met and low Met/Phe from 0.50 to 0.53 MoM, and 0.55 to 0.60 MoM, respectively, maintained the same

sensitivity of 0.88; this modification decreased substantially the number of second-tier tests of tHcy from 1.8 % to 0.9 % and the costs from € 39,867 to € 16,639/year. In this small study, the modified approach for the model-tested NBS program yielded a sensitivity of 1. for CBSD and 0.88 for iRMD with less demand for second tier tHcy testing leading to savings of € 22,404 per year.

Discussion

The long-term evidence on favourable clinical outcome in early treated patients strongly indicates that CBSD is a good candidate for NBS (Gramer et al 2017; Huemer et al 2015, Okun et al 2016, Morris et al 2017). NBS for RMD should at least be considered (Huemer 2015, Huemer 2017, Weisfeld-Adams et al 2010, 2013; Martinelli et al 2011, Carrillo-Carrasco et al 2011) since MTHFR deficiency seems to be particularly responsive to early betaine treatment (Diekman et al 2014) and the majority of early treated cobalamin-related RMD have a more favourable clinical outcome in terms of less mortality and severe organ complications when treated early. However, brain and eye disease may progress in RMD (especially in the cblC defect). The recommendation on MATI/IIID as a target condition are controversial, mostly due to detection of large numbers of heterozygotes (Huemer et al 2015).

This study involving 18 countries shows however, that NBS practice is not concordant with these recommendations. Despite some evolution since 2011 (Loeber et al 2012), NBS for the homocystinurias is not widely established.

The decision on disorders to be included into NBS programs is only in part evidence-based. The final program setup is a political decision of states, regions and institutions and this in fact explains the heterogeneity among different regions and countries. In the USA, the Advisory Committee on Heritable Disorders in Newborns and Children (https://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/recommendedpanel/) recommended screening for CBSD as a primary target, and RMD and MATI/IIID as secondary conditions. In the EU, the absence of harmonized recommendations is reflected by the wide range of none to seven homocystinurias targeted in different European NBS programs. Interestingly, there is considerable variation of types of homocystinurias included in NBS panels even between regions within one country, reflecting different decision-making routes.

Furthermore, this study showed a large variability in decision limits between individual programs and demonstrated that some programs use decision limits with suboptimal sensitivity. Only a small proportion of programs use second tier strategies that would allow implementation of decision limits with higher sensitivity.

The reasons for differing decision limits among centres are several. A main influence may be varying sampling times against the background of the physiological changes of marker concentrations during the first days of life. In the Collaborative Laboratory Integrated Reports (http://clir.mayo.edu) changes of markers after birth are apparent for Met (median 23 µmol/

at 24 hours and 18.5 µmol/L at 48 hours) and less strikingly for Met/Phe (0.45 at 24 hours and 0.40 at 60 hours), and C3/C2 (0.074 at 48 hours and 0.085 at 60 hours). Furthermore, most probably the different performance of the various analytical programs in place add to the heterogeneity we observe. This effect is demonstrated by the CDC Newborn Screening Quality Assurance Program. Data from CDC on Set 2 July—October 2016 (CDC 2017) show that between seven different types of analytical platforms the mean Met concentrations in a single DBS batch varied between 17.6 and 24.1 µmol/l.

The effect of different sampling times and platforms may be minimized by using multiples of reference medians (MoM). Nevertheless in this study, use of MoM-corrected values did not decrease the huge variability of decision limits among centres. The reasons for this lack of concordance is unknown and may include the use of different metrics (e.g. different percentiles of reference population used as cut-offs), various degrees of acceptance of false positivity or different follow-up procedures in individual programs.

Since sensitivity of markers to detect homocystinurias is inversely related to specificity, second tier testing allows for adoption of more sensitive decision limits without causing a massive increase of false positive results which result in recalls, consecutive psychological stress for families, and increased costs (Gramer et al 2017, Karaceper et al 2016). Gramer et al (2017) have proposed an algorithm to detect CBSD and RMD with tHcy as a second-tier test in all newborns with abnormalities in either Met (lower cut-off only) or Met/Phe (lower and upper cut-off) or elevated C3 or C3/C2. This approach was tested in a single centre and did not only identify patients from the local population but also patients documented in the Region-4-Collaborative Project (Gramer et al 2017). The authors show that second tier measurement of tHcy allows adjusting decision limits of the primary parameters towards the median, which results in a smaller proportion of patients being missed. These and similar recommendations to use tHcy and/or MMA as second-tier markers (Huemer 2015) have not yet been widely adapted in NBS practice as demonstrated by our study. Consequently, many NBS programs for homocystinurias miss the opportunity of establishing more sensitive decision limits or probably produce unnecessarily high numbers of false positive results.

This study has several limitations. It is not entirely representative as only centres with particular interest in homocystinurias and thus participating in the E-HOD registry had been invited.

With the exception of seven cblC/cblD patients missed by NBS, all data on marker values come from patients detected by NBS. Consequently, marker values from patients with milder form of disease will most probably be underrepresented in the dataset which in turn probably leads to a biased evaluation of decision thresholds. Unfortunately false negative cases are not easily detectable and the retrospective analyses of stored DBS from patients with milder or late onset forms of homocystinurias is hampered by instability of some markers, ethical concerns or the early destruction of DBS according to local or national policies.

Conclusions and recommendations

This study demonstrates that many NBS programs have not yet implemented the recent recommendations on screening for the homocystinurias. We recommend to include homocystinurias into NBS panels. We propose to re-evaluate decision limits, combine relevant parameters and to adopt strategies based on the median of the local reference population. Determination of tHcy as primary marker is not widely used due to pre-analytical challenges, longer instrument time and substantial costs. We strongly recommend using tHcy and MMA as second tier markers to allow for more sensitive decision limits, increase specificity and lower costs of NBS programs for homocystinurias.

Acknowledgements

We thank Professor Burkhardt Seifert, Dept. of Biostatistics, University of Zürich for statistical advice, Erica L. Wright for providing information on the Colorado and Wyoming screening program and Marike Groenendijk (EHOD project) for her continuous support. PCh, JB, MP and VK received institutional support from the Ministry of Health (project DRO VFN64165) and from the Charles University (project PROGRES Q26). This publication arises from the project E-HOD (Chafea grant no. 2012 12 02) which has received funding from the European Union, in the framework of the Health Programme.

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