Role of IgM testing in the diagnosis and post-treatment follow-up of syphilis: a prospective cohort study

Kara K Osbak, Achilleas Tsoumanis, Irithe De Baetselier, Marjan Van Esbroek, Hilde Smet, Chris R Kenyon, Tania Crucitti

ABSTRACT


Methods This substudy was conducted in the context of a syphilis biomarker discovery study (ClinicalTrials.gov Nr: NCT02059525). Sera were collected from 120 individuals with a new diagnosis of syphilis (72 with repeat infections) and 30 syphilis negative controls during a cohort study investigating syphilis biomarkers conducted at a sexually transmitted infection/HIV clinic in Antwerp, Belgium. Syphilis was diagnosed based on a simultaneous positive treponemal and non-treponemal assay result and/or positive serum PCR targeting polA. Specimens collected at visit of diagnosis, and 3 and 6 months post-treatment were tested by two enzyme immunoassays (EIAs), recomWell (Mikrogen; MI) and Euroimmun (EU), to detect anti-treponemal IgM. Baseline specimens were also tested for anti-treponemal IgM using a line immunoassay (LIA) recomLine (MI). Quantitative kinetic decay curves were constructed from the longitudinal quantitative EIA results.

Results An overall sensitivity for the diagnosis of syphilis of 59.8% (95% CI: 50.3%–68.7%), 75.0% (95% CI: 66.1%–82.3%) and 63.3% (95% CI: 54.8%–72.6%) was obtained for the EU, MI EIAs and MI LIA, respectively. When only considering repeat syphilis, the diagnostic sensitivity decreased to 45.7% (95% CI: 33.9%–58.0%), 63.9% (95% CI: 51.7%–74.6%) and 47.2% (95% CI: 35.5%–59.3%), respectively. IgM seroreverted in most cases 6 months after treatment. Post-treatment IgM concentrations decreased almost 30% faster for initial syphilis compared with repeat infection. The IgM EIAs and IgM LIA agreed from fairly to moderately (Cohen’s kappa (κ): 0.36 (EU EIA); κ: 0.53 (MI EIA); κ: 0.40 (MI LIA)) with the diagnosis of syphilis.

Conclusions IgM detection was not a sensitive method to diagnose syphilis and was even poorer in the diagnosis of syphilis repeat infections.

INTRODUCTION

Syphilis has re-emerged during the last 15 years as a major public health problem with increasing incidence particularly among men who have sex with men (MSM) in the northern hemisphere. It is a multistage chronic disease caused by Treponema pallidum subspecies pallidum, and can be challenging to diagnose, especially very early and repeat infections. Diagnostic strategies remain reliant on serological testing, however, deficiencies in assay performance and persistent anti-treponemal antibody presence following initial infection often hamper diagnostic accuracy.

As the epidemics progress, an increasing proportion of all infections have been noted to be reinfections. Since reinfections are more likely to present asymptomatically, timely diagnosis depends on the diagnostic accuracy of serological tests.

Since treponemal tests (TT) remain positive for life, the diagnosis of reinfections typically depends on fourfold or greater rises in non-treponemal test (NTT) titres, such as the rapid plasma reagin (RPR) test. Conversely, a fourfold decline in NTT titres is used to determine the success of syphilis treatment.

Strengths and limitations of this study

- The clinical utility of IgM detection was investigated for the diagnosis of syphilis and post-treatment follow-up in a prospective cohort of 120 individuals with a new episode of syphilis (72 with repeat infections) and 30 syphilis negative controls.
- Syphilis was diagnosed based on a simultaneous positive treponemal and non-treponemal assay result and/or positive serum PCR targeting polA.
- Sera were collected at visit of diagnosis, and 3 and 6 months post-treatment were tested by two different commercially available enzyme immunoassays to detect anti-treponemal IgM, whereby quantitative kinetic decay curves were constructed from the longitudinal quantitative results.
- Specimens collected at diagnosis were also tested for anti-treponemal IgM using a line immunoassay.
- The study population was mostly HIV-infected men who have sex with men taking antiretroviral therapy, therefore the possible effects of HIV infection such as viral load and CD4+ T cell count and gender were not controlled for.
Due to the aspecific nature of NTTs, that relies on antibo-
dy binding to lipoidal components released during
host cell destruction and also present in low quantities
in the T. pallidum cell wall, other biological, infectious
or immunological mechanisms can also result in fluctua-
tions in NTT leading to false positive results.

Following an initial infection, immunoglobulin (Ig)M
antibodies are the first class of antibodies produced. Early
detection of IgM could therefore help with the diagnosis
of early syphilis. Current European syphilis guidelines
mention that IgM testing is useful in the assessment of
newborns and cerebral spinal fluid. These guidelines also
note that a negative IgM result cannot exclude the diag-
nosis of congenital or neurosyphilis. There is however
little published on the clinical utility of IgM testing in
these contexts. A study published in 2013 evaluated
three commercially available IgM enzyme immunoas-
says (EIAs) using 307 serum samples from individuals
with active syphilis. It found that these IgM assays had a
median sensitivity of 84.5% with specificities in the range
of 91.4%–100%. In 23/59 (39%) of cases the IgM EIA
test was positive in suspected very early infection where
the NTT was negative. Even less has been published in
the context of the current outbreaks of syphilis. In many
countries a large proportion, and sometimes most syphilis
cases, are repeat episodes of syphilis. IgM responses are
frequently different in repeat episodes of infection. We
could only find one paper that has evaluated the utility
of IgM testing for the diagnosis of repeat syphilis. This was
a prospective analysis of IgM among a cohort of MSM. It
found a low performance of the EIA IgM test for repeat
syphilis diagnosis, namely only 38.5% of repeat syphilis
cases were diagnosed correctly with IgM testing. The
number of patients with repeat syphilis in the study was
only 13.

In this study we investigated the clinical utility of IgM
detection in the diagnosis of syphilis and post-treatment
follow-up in a prospective cohort of 120 individuals with
a new episode of syphilis. In addition, we evaluated the
performance of three commercially that is, two EIAs and
one line immuno assay (LIA), IgM assays.

MATERIALS AND METHODS

Study design

Potentially eligible study participants 18 years or older,
in whom a new syphilis diagnosis was made, were consec-
cutively screened and prospectively recruited between
January 2014 and August 2015 at a sexually transmitted
infection (STI)/HIV clinic in Antwerp, Belgium. Study
exclusion criteria were the use of beta-lactam, doxy-
cycline or macrolide antibiotics during the 28 days
preceding enrolment. Syphilis diagnosis and disease
staging were performed by the study physician according
to the Centers for Disease Control guidelines. Infection
was defined as an episode of syphilis that followed
a previously clearly documented episode of syphilis. Stage-appropriate treatment was administered according
to European guidelines. This involved intramuscular
benzathine penicillin 2.4 mu weekly for 3 weeks for late
latent syphilis and a stat dose for primary, secondary and
eyear latent syphilis. All participants with syphilis were
followed-up by the study team at the STI clinic 3 and 6
months post-treatment. The study physician recorded
clinical details and laboratory results in a standardised
fashion during each study visit. HIV-infected controls with
both negative NTT and TT results were included during
the same recruitment period at the same study location.
Clinical details were recorded for the controls during a
single clinical visit, followed by a same day blood draw.

Patient and public involvement

Patients or the public were not involved in the design of
the study.

Clinical serological testing during routine workout

Blood was drawn into serum gel tubes (Sarstedt Monovette,
Nümbrecht, Germany). Sera were divided and either (1)
stored at 4°C–8°C until routine syphilis serological testing
within 4 days or (2) stored at −80°C within 3 hours for later
testing. Routine serological testing included Macro-Vue
RPR Card (Becton Dickinson, Sparks, Maryland, USA) and
TPA assay (Ortho-Clinical Diagnostics, Rochester, New
York, USA) testing following the manufacturer’s
instructions. Positive RPR results were determined to a
titre endpoint.

Sera obtained at baseline were also tested with the
SERODIA-T. pallidum particle agglutination (TP-PA)
(Fujirebio, Tokyo, Japan) assay and an in-house T. pall-
idum PCR targeting polA.

EIA and LIA testing for IgM serum antibodies

In order to investigate if assays from different manufac-
turers or using another method performed differently
and thus would have an impact on our study outcome, we
evaluated side by side two IgM EIAs and one LIA.

The anti-T. pallidum IgM EIAs were provided by Euro-
immun (Lübeck, Germany) and by Mikrogen GmbH
(Reutried, Germany), henceforth referred to as ‘EU EIA’
and ‘MI EIA’, respectively. Both assays use microplate
wells coated with a mixture of four antigens of T. pallidum:
Tp15, Tp17, Tp47 and TmpA. Testing was performed
following the manufacturer’s instructions (EI_2111M_-
A_UK_C07.doc; V.08/09/2011 and GIRETP011DE.doc;
V.April 2010). All quantitative EIA results were expressed
in International Units (IU)/mL. The lower detection limit
of the EU EIA was defined as the ratio value of 0.06 by the
manufacturer. A ratio result of ≥0.8 to <1.1 was defined as
borderline. The results were semi-quantitatively evaluated
by calculating a ratio of the extinction value of the patient
sample over the extinction value of the calibrators. The
manufacturer reported a sensitivity and specificity of
100%, with an intra-assay variation (CV) of 4.17% and
inter-assay CV of 5.3%. With regards to the MI EIA, the
manufacturer reported sensitivity was 100%, intra-assay
CV 4.5% and inter-assay CV <11%. Borderline values were defined as ≥20–≤241U/mL.

The recomLine Treponema LIA kit (MI LIA) (Mikrogen GmbH) was used for the qualitative determination of the IgM antibodies for baseline samples. This test uses recombinant T. pallidum antigens fixed on nitrocellulose membrane strips. Testing was performed according to the manufacturer’s instructions (GARLTP002EN, V2012/08). Objective reading of the strips was done by scanning the strips with a flatbed scanner and analysis software Recomscan (Mikrogen). The density of the antigen was compared with a positive control and ratios were calculated. The assay was reported negative if no antigen was detected (ratio <1), borderline if only one random antigen was detected (ratio ≥1) and positive if at least two random antigens were present (ratio ≥1). The manufacturer reported sensitivity was 100% and specificity 95%.

Quality control
The samples were analysed by a research and diagnostic laboratory unit, both are ISO15189 accredited. One single lot number was used for all EIAs and LIAs. Two laboratory technicians performed the analyses during two batch testing periods of 14 days in 2016. Evaluative testing of the EIAs took place maximum 974 days after the samples of interest were collected. Samples were thawed, tested and refrozen on the same day with a maximum of two freeze thaw cycles.

Due to the prolonged testing period and nature of the routine diagnostic laboratory setting, the reagents used for the TPPA, TPA and RPR tests were from different lot numbers.

The laboratory technicians were blinded from the patient’s clinical information and any other syphilis serology result.

Data were manually double entered into the database.

Definitions
In the absence of a single gold standard test for syphilis, a positive RPR test, or in the case of reinfection a four-fold increase in RPR titre, together with a positive TPPA/TPA results or a positive T. pallidum PCR test were used to define a new syphilis episode (‘syphilis diagnosis’).

Statistical analysis
Percentage agreement and Cohen’s kappa (κ)-coefficient value were calculated to estimate agreement between the IgM test results. The sensitivity, specificity, positive and negative predictive values with 95% CIs were calculated using the baseline visit samples. Borderline results for the EIA and LIA testing were included for the first set of analyses as negative (specific scenario) and the second dataset included all borderline results as positive (sensitive scenario).

Continuous variables were expressed as median values and IQR. Associations between categorical variables were assessed with the χ² test and Fisher’s exact test for small numbers. Mann-Whitney U test was performed to compare the quantitative results of the IgM assays between initial and repeat syphilis groups, in addition to symptomatic (primary and secondary stage) versus latent syphilis groups. An all-available case approach was used throughout all analyses. Analyses were performed in Stata V.13.1 (StataCorp, College Station, Texas, USA). The statistical significance level was set at 0.05.

Non-linear models were used to visually assess the decay of the IgM concentrations and RPR titres over time. The exponential decay curves were of the form \( Y = a e^{bX} \), where \( Y \) was the measurement of the EIA tests and \( X \) the time in months. The exponential decay curve and the graphics were designed using R V.3.4.4 and the package nlstools.17

RESULTS
Study participants
In total 150 individuals were included in the study, 120 diagnosed with syphilis and 30 controls.18 Study subject characteristics are described in table 1. Of those who had active syphilis at the time of study enrolment 48/120

<table>
<thead>
<tr>
<th>Table 1 Study subject characteristics</th>
</tr>
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<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
</tr>
<tr>
<td>MSM</td>
</tr>
<tr>
<td>HIV-infected</td>
</tr>
<tr>
<td>Taking ART</td>
</tr>
<tr>
<td>Benzathine penicillin G treatment</td>
</tr>
<tr>
<td>RPR-C titre at baseline</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>CD4+ T cell count</td>
</tr>
</tbody>
</table>

*Control subjects were all HIV positive and non-treponemal and treponemal antibody negative at the time of study inclusion.
†Statistically significant difference between syphilis positive group and controls.
‡Percentage calculated with denominator n=120 syphilis-positive subject.
ART, antiretroviral therapy; MSM, men who have sex with men; NA, not applicable; RPR, rapid plasma reagin.

(40%) presented with a first episode of syphilis (henceforth referred to as ‘initial infection’) and 72/120 (60%) presented with a repeat infection. Previous NTT results were available for 107/120, the remaining 13 individuals without previous serological test results were classified as having an initial infection based on the fact that they had never had a diagnosis of or symptoms suggestive of syphilis before. Although initial infections were more often symptomatic 34/48 (71%) compared with the repeat infections 39/72 (54%), this difference did not reach statistical significance (p=0.09).10

**IgM test results**

The IgM testing was performed on 339, 343 and 150 sera by the EU, MI EIAs and LIA, respectively. Details of the qualitative results are presented in table 2. One sample from a control patient tested IgM positive and another one tested borderline positive with the MI EIA. The LIA also had one false positive and one borderline result. All control samples tested negative with the EU EIA. Slight differences in the number of samples tested per visit (baseline N=3 and M6 N=1) between the two EIAs was due to logistical error. Samples were not available for all follow-up visits due to non-attendance; the reason was not recorded.

**IgM test performs sub-optimally for diagnosis of syphilis**

The overall diagnostic performance for the two IgM EIAs and LIA assays evaluated was moderate. Table 3 summarises the assay performances in the specific-case scenario. The diagnostic sensitivities of the evaluated assays ranged from 80.9% to 91.7% when applied to samples collected at baseline from individuals with initial syphilis. When baseline analyses were stratified per initial or repeat infection, the diagnostic sensitivity decreased significantly in repeat infections for all assays evaluated or repeat infection, the diagnostic sensitivity decreased significantly in repeat infections for all assays evaluated.

**Quantitative assessment of baseline samples with EIA**

When considering the quantitative results of the two EIAs, a significant difference was found between the initial and repeat syphilis groups (EU p=0.0000; MI p=0.0002) with repeat syphilis having lower IgM concentrations. Moreover, the concentrations were significantly lower for individuals presenting with latent stage syphilis compared with primary and secondary stage (EU EIA p=0.001; MI EIA p=0.0002).

**Agreement between the commercial IgM tests**

The overall agreement was substantial between the two EIA tests when considering all samples that is, samples from baseline, M3 and M6, tested by both assays (N=339). The Cohen’s kappa was κ: 0.69 (85.3%) and κ: 0.74 (86.7%) for the specific-case and sensitive-case scenarios, respectively.

There was a strong agreement between the LIA and the EIAs: 90.7% (κ: 0.81) between the MI EIA and MI LIA, and 95.3% (κ: 0.91) between EU EIA and MI LIA in the specific-case scenario. In the sensitive-case scenario the agreement between MI EIA and MI LIA increased further up to 99.3% (κ: 0.91) but decreased between EU EIA and MI LIA to 88.7% (κ: 0.77).

**DISCUSSION**

We aimed to determine whether testing for IgM could aid in the diagnosis of new syphilis infections, including repeat infections, and if it could be useful for post-treatment follow-up. Detection of IgM may help in the diagnosis of syphilis but its diagnostic sensitivity is poor, it was notably low in participants diagnosed with repeat syphilis. Our results are, however, congruent with previous reports of suboptimal IgM test performance overall and for repeat syphilis in particular.11 The lower diagnostic sensitivity of IgM in repeat syphilis is compatible with results from other infections where reinfections lead directly to increases in IgG without initial increases in IgM.10

This study represents the most comprehensive evaluation of EIA and LIA IgM testing on serum from individuals with syphilis that we are aware of. We found that both EIA agreed substantially, although that the MI EIA performed in terms of diagnostic sensitivity better than the EU assay. The difference between both sandwich EIAs lays in the pre-treatment of serum with the IgG/rheumatoid factor (RF) absorbent included in the EIA reagent kit of EU. The absorbent removes the antibodies of the IgG classes and the RFs, known to be possible
Table 2  Qualitative enzyme immunoassay and line immunoassay results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EU EIA</th>
<th>MI EIA</th>
<th>MI LIA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total N</td>
<td>Positive N (%)</td>
<td>Borderline N (%)</td>
</tr>
<tr>
<td><strong>Time of testing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>147</td>
<td>70 (48)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>3M</td>
<td>86</td>
<td>16 (19)</td>
<td>10 (12)</td>
</tr>
<tr>
<td>6M</td>
<td>106</td>
<td>16 (15)</td>
<td>6 (6)</td>
</tr>
<tr>
<td><strong>Syphilis stage/controls at baseline visit</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>23</td>
<td>14 (61)</td>
<td>3 (13)</td>
</tr>
<tr>
<td>Primary (repeat only)</td>
<td>11</td>
<td>5 (46)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Secondary</td>
<td>48</td>
<td>33 (69)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Secondary (repeat only)</td>
<td>27</td>
<td>13 (48)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Early latent</td>
<td>30</td>
<td>14 (47)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Early latent (repeat only)</td>
<td>23</td>
<td>10 (43)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Late latent</td>
<td>16</td>
<td>9 (56)</td>
<td>2 (13)</td>
</tr>
<tr>
<td>Late latent (repeat only)</td>
<td>9</td>
<td>4 (44)</td>
<td>2 (22)</td>
</tr>
<tr>
<td>Controls</td>
<td>30</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Syphilis history</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial infection</td>
<td>47</td>
<td>38 (81)</td>
<td>4 (9)</td>
</tr>
<tr>
<td>Repeat episode</td>
<td>70</td>
<td>32 (46)</td>
<td>5 (7)</td>
</tr>
</tbody>
</table>

1. The number of baseline samples includes the 30 samples of the control syphilis-negative individuals.
2. The number of positive MI EIA and MI LIA results includes one positive result obtained among the control samples.
3. The number of borderline MI EIA and MI LIA results includes one borderline result obtained among the control samples.

EIA, enzyme immunoassay; EU, Euroimmun; LIA, line immunoassay; MI, Mikrogen; NT, not tested.
interferences in IgM EIA. False positive reactions may occur in pathogen-specific IgM detection when the IgM RFs bind to IgG immune complexes. On the other hand, false negative results may appear when pathogen-specific IgM antibodies are displaced by stronger binding IgG.19 We cannot rule out that this extra IgG/RF removal step may have contributed to the differences in test performance found between both EIAs.

The general performance of the LIA for the baseline syphilis diagnosis was similar to the EIAs. The antigen line reaction varied by sample and in some samples only a few antigens could be demonstrated. Since no particular single antigen or line can be attributed to a stage or profile, an overall qualitative result should be considered.

In the majority of the IgM positive cases detected at baseline, the IgM antibodies disappeared 6 months after...
treatment. Interestingly, the decline in IgM concentrations occurred faster in initial syphilis compared with repeat syphilis. Our study hints but remains inconclusive and additional studies are required to investigate whether IgM concentration measurements may be a more objective, useful and high throughput method compared with RPR for the follow-up of syphilis treatment.

More studies are needed to investigate the role of IgM in syphilis diagnosis on a more diversified and larger scale (eg, women, non-MSM populations). Since our study only included individuals with a dual positive NTT/TT we were unable to evaluate if IgM values might precede NTT/TT seroconversion. A previous study did find that IgM could be useful in the early diagnosis of syphilis in the subset of patients with equivocal TT and negative NTTS.9

Strengths of this study include the comprehensive characterisation of sera and its prospective nature. Limitations to this study include the fact that the study population was mostly HIV infected. We did not control for possible effects of HIV infection such as viral load and CD4+ T cell count, although notably most HIV-infected participants were taking antiretroviral therapy and had a high immune cell count thus the likelihood of this having an effect on the test outcome is unlikely. The study was not designed to adequately evaluate the assays’ specificity.20 Previous serology results were not available for 13 patients. For this group syphilis history was reliant on patient recall of clinical symptoms. It is possible that some individuals unknowingly had a previous syphilis episode, therefore the number of reinfections could be higher than reported. This could possibly affect IgM performance results. Moreover, follow-up sera samples were not available for all 3M and 6M study participants, however, most (88%) endpoint M6 samples were analysed.

CONCLUSIONS
In conclusion, the overall diagnostic performance for the two IgM EIAs and LIA assays evaluated was moderate. When IgM results were stratified per initial or repeat infection, the diagnostic sensitivity decreased significantly in repeat infections for all assays evaluated. This could lead to missed infections. Individuals with repeat syphilis or presenting with latent stage syphilis had lower IgM concentrations compared with initial infection and primary/secondary stage syphilis, respectively. Future research could evaluate the utility of IgM in the follow-up of treatment. For example, serial IgM testing may play a role in the management of serofast syphilis.
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Contributors KKO, IDB, MVE, CRK and TC conceived the study. HS and KKO coordinated and performed laboratory analyses and entered data. MVE, IDB and TC supervised laboratory activities. KKO and AT managed and conducted the data analyses. KKO wrote the first draft. All authors contributed to the final version of the manuscript and approved the final manuscript.

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Treponema pallidum IgM assays were provided free of charge by Euroimmun (Lübeck, Germany) and by Mikrogen GmbH (Neuried, Germany).

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Ethics approval The Institutional Review Board of the ITM and the Ethics Committee of the University Hospital Antwerp approved this study (13/44/426). Written informed consent for study participation and reporting of anonymised clinical details was obtained from all participants upon study inclusion.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The data supporting the findings of this publication are retained at the Institute of Tropical Medicine (ITM), Antwerp and will not be made openly accessible due to ethical and privacy concerns. According to the ITM research data sharing policy, only fully anonymised data can be shared publicly. The data are de-identified (using participant identification numbers only) but not fully anonymised and it is not possible to fully anonymise them due to the longitudinal nature of the data. Data can however be made available after approval of a motivated and written request to the ITM at ITMresearchdataaccess@itg.be. The ITM data access committee will verify if the dataset is suitable for obtaining the study objective and assure that confidentiality and ethical requirements are in place.

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ORCID iDs
Irlith De Baetselier http://orcid.org/0000-0002-1804-252X
Tanja Crucitti http://orcid.org/0000-0002-2235-6038

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