Overexpression of *plasmepsin II* and *plasmepsin III* does not directly cause reduction in *Plasmodium falciparum* sensitivity to artesunate, chloroquine and piperaquine

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**ABSTRACT**

Artemisinin derivatives and their partner drugs in artemisinin combination therapies (ACTs) have played a pivotal role in global malaria mortality reduction during the last two decades. The loss of artemisinin efficacy due to evolving drug-resistant parasites could become a serious global health threat. Dihydroartemisinin-piperaquine is a well tolerated and generally highly effective ACT. The implementation of a partner drug in ACTs is critical in the control of emerging artemisinin resistance. Even though artemisinin is highly effective in parasite clearance, it is labile in the human body. A partner drug is necessary for killing the remaining parasites when the pulses of artemisinin have ceased. A population of *Plasmodium falciparum* parasites in Cambodia and adjacent countries has become resistant to piperaquine. Increased copy number of the genes encoding the haemoglobinases Plasmepsin II and Plasmepsin III has been linked with piperaquine resistance by genome-wide association studies and in clinical trials, leading to the use of increased plasmepsin II/plasmepsin III copy number as a molecular marker for piperaquine resistance. Here we demonstrate that overexpression of *plasmepsin II* and *plasmepsin III* in the 3D7 genetic background failed to change the susceptibility of *P. falciparum* to artemisinin, chloroquine and piperaquine by both a standard dose-response analysis and a piperaquine survival assay. Whilst plasmepsin copy number polymorphism is currently implemented as a molecular surveillance resistance marker, further studies to discover the molecular basis of piperaquine resistance and potential epistatic interactions are needed.

**1. Introduction**

Intraerythrocytic malaria parasites propagate successfully inside the human host by devouring a vast amount of haemoglobin. This is achieved by digesting the haemoglobin proteins by proteases in the food vacuole (Francis et al., 1997b). The digestive process employs a cascade of haemoglobinases cooperating to process various globins into short peptides (Klemba and Goldberg, 2002). The released haem molecules are not catabolised but instead are packed into relatively inert semicrystalline structures (haemozoin or malaria pigment) to prevent...
oxidative damage from iron-containing haem moieties (Sigala and Goldberg, 2014). The haemoglobinases work together as a large multi-subunit protein complex (Chugh et al., 2013). In *Plasmodium falciparum*, this haemoglobin-digestion complex is composed of three groups of proteases namely, falcipain, falcipain and plasmpesin (Goldberg, 2005). The evolutionary history of these gene families reveals their specialized roles in the *Plasmodium* species as reflected by their respective independent gene expansion events (Ponsuwanna et al., 2016). This protein complex is associated with the actions of several antimalarial drugs including the quinoline related antimalarials (quinine, chloroquine, mefloquine etc) and artemisinin (Chugh et al., 2013). Genetic variations in these genes are also associated with changes in drug susceptibility (Amato et al., 2017; Klonis et al., 2011; Witkowski et al., 2017).

Recently, *P. falciparum* in Cambodia has started to lose sensitivity to piperaquine (Amaratunga et al., 2016; Chaoarattanakavee et al., 2016), a key partner in one of the artemisinin combination therapy regimens. Piperaquine belongs to the 4-aminoquinoiline chemical group, a class which includes chloroquine and amodiaquine (Pussard and Verdier, 1994). It retains activity against chloroquine resistant *P. falciparum* (Pascual et al., 2012). Piperaquine is a bisquinoline containing a bis-piperazine propane connecting bridge (Raynes, 1999). Dihydroartemisinin-piperaquine combination therapy was implemented in Southeast Asia as an alternative to the artesunate-mefloquine regimen (Woodrow and White, 2017). Choosing the appropriate partner drug for artemisinin is an important public health issue since the life-saving artemisinin derivatives have short half-lives in the human body and require a partner drug with a considerably longer half-life to ensure complete parasite clearance and thus prevent the emergence and spread of drug resistance (Eastman and Fidock, 2009). Reduced artemisinin susceptibility leads to decreased parasite killing and a much greater residual of parasites for the partner drug to remove, which increases the selective pressure on partner drug resistance.

Plasmpesins have become a topic of interest because of two independent genome-wide association studies (GWAS) showing variations in gene copy as a marker of piperaquine resistance (Amato et al., 2017; Witkowski et al., 2017). Plasmpesins belong to the aspartic protease group, which employs a catalytic dyad of two aspartic residues (Silva et al., 1996). There are ten Plasmpesin proteases in *P. falciparum* (Plasmpesin I to Plasmpesin X) (Ponsuwanna et al., 2016). Only Plasmpesin I - IV function in haemoglobin digestion, and the remaining enzymes have diverse biological functions (Ponsuwanna et al., 2016). For example, Plasmpesin V processes malarial proteins for transport from the intra-erythrocytic parasite to the red blood cell cytosol (Boddey et al., 2013; Sleeb et al., 2014). Interestingly, the genes encoding the haemoglobin-specific Plasmpesin proteins are located in the same gene cluster on chromosome 14 (Coombs et al., 2001). The expansion of the haemoglobin-specific plasmpesin genes occurred after the diversification of Plasmpesin functions (Ponsuwanna et al., 2016). Plasmpesin III is called Histo-Aspartic Protease (HAP) because its active site contains a dyad of histidine and aspartic acid residues instead of the same gene cluster on chromosome 14 (Coombs et al., 2001). The ex-

2. Material and methods

2.1. Overexpression of plasmpesin genes

The coding sequences of plasmpesin II (PF3D7_1408000) and plasmpesin III (PF3D7_1408100) genes were PCR amplified from genomic DNA of *Plasmodium falciparum* 3D7 using primer pairs containing reverse enzyme sites at 5′ and 3′ ends; PM2F 5′-ACAGGGATCCATG GATATTACAGAAGAGACAT-3′ and PM2R 5′-CGGCGAGCTTAGTTTA TAAAATCTTTTAGAAAG-3′ (AvrII and SpeI give the same overhang site); PM3F 5′-CTTAGGATCCATGATTTAACATTAAAAGA-3′ and PM3R 5′-CCTCAGTGTATTTAAATTTTGACTAAG-3′, respectively. The ampiclon was cloned into the pBS dep20 vector using the BamHI and SpeI sites (Sato et al., 2003; Wittayacom et al., 2010). The plasmid constructs were verified by DNA sequencing. The plasmid was transformed into the *P. falciparum* 3D7 strain using the DNA-loaded red blood cell method (Deitsch et al., 2001). Briefly, red blood cells were washed with cytostix (120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄ and 25 mM HEPES, pH 7.6). The mixture of plasmid DNA and red blood cells was electroporated by using Bio-rad Gene Pulser (0.31 kV and capacitance 960 μF). The loaded red blood cells were added into the culture with fresh schizont-infected red blood cells. The parasites were maintained in standard complete medium (RPMI 1640, 25 mM HEPES, 0.2% NaHCO₃, 0.5% albumax, 146.9 μM hypoxanthine and 25 μg/mL gentamicin) under 2 μg/mL blastidicin (Gibco) selection, and emerging parasites were checked for the plasmid by PCR amplification using the bsd gene with primers bsd-F 5′-AGGTCCTTCGTCAGTCCGAT-3′ and bsd-R 5′-CATACAAC GAGGCGAGCAT-3′ (Mamoun et al., 1999).

The RNA expression of plasmpesin II and plasmpesin III in transgenic parasites was analyzed by using RT-qPCR. The parasite lines were tightly synchronized by two consecutive rounds of 5% sorbitol treatment. Trophozoites (24–32 h after reinfection) were collected for RNA extraction. plasmpesin II, plasmpesin III and calmodulin (PF3D7_1434200, the promoter driving the expression) are all highly expressed in this window (Otto et al., 2010). Every RT-qPCR reaction was performed at least in duplicate in two independent experiments. The raw cycle threshold (Ct) values of PM 1, PM 2, PM 3 and PM 4 with the no reverse transcriptase control and fold change calculation are shown in Supplementary Table 1. Total RNA was extracted by using Trizol reagent (Ambion) and sequentially treated with RNase-free Dnase I (Promega). Superscript III First-Strand Synthesis System (Invitrogen) was used to convert RNA to cDNA with random hexamer primers (Invitrogen). qPCR reactions were performed using KAPA SYBR® Fast qPCR kit master mix, according to the manufacturer’s recommended conditions at 95 °C for 3 s, 55 °C for 20 s and 72 °C for 20 s (35 cycles) with the Mastercycler® ep realplex (Eppendorf). Four plasmpesin genes, PM 1 (PF3D7_1407900), PM 2, PM 3 and PM 4 (PF3D7_1407800), were quantified by comparing with Actin I (PF3D7_1246200), an endogenous control gene in *P. falciparum* 3D7 (blank vector control) and *P. falci-

parasites. The primer information is as follows: PM1-F 5′-AGGTAATGCTGGTATAGTGGAA-3′, PM1-R 5′-CAATTGACCTTGGGAAACCA-3′, PM2-F 5′-TGGTGATCGAAGATTGGAG-3′, PM2-R 5′-TCTTCAGCTGGTGAATTTAACAC-3′, PM3-F 5′-AGATCCCTTCAACACGGTTCGGG-3′, PM3-R 5′-CACCTTCGCAAACATCATGAA-3′, PM4-F 5′-TGCGTCTTACCATTAAAGAGA-3′, PM4-R 5′-CACACCCGTAATAAGGCAACA-3′, Actin-F 5′-AGGACCGAAGATTCCACACA-3′ and Actin-R 5′-GATGTTGCAAGGGTGTGAA-3′. The fold change of gene expression was calculated by using the 2−ΔΔCT method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Controls with no reverse transcriptase were included to monitor genomic DNA contamination. Student’s t-test was used for statistical testing.

Western analysis was performed with the parasite extract prepared from 3% synchronous trophozoites. Parasitised red blood cells were lysed with 0.15% saponin on ice for 1 min. The parasite pellets were washed twice with Phosphate Buffer Saline (PBS) containing protease inhibitor cocktail cOmplete™ (Roche). The parasite pellets were heated in NuPAGE LDS sample buffer (Thermo Fisher Scientific) and were analyzed in 4–12% Bis-Tris NuPAGE Gel (Thermo Fisher Scientific). The protein was transferred onto Hybond ECL™ nitrocellulose membranes (GE Healthcare) and subsequently blocked with 5% milk. The blots were incubated with 1:3000 polyclonal anti-plasmepsin II (1:3000) and prepared by two-fold serial dilutions in 96-well plate. The drug concentrations were varied (1 μM-1 nM). The IC50 determination for artemesunate was performed by using a newly modified method based on the Trophozoite Maturation Inhibition assay (TMI) (Chotivanich et al., 2014). Lactate dehydrogenase-based Inhibitory Concentration Assay (LICA) was developed in order to reduce the subjective read-out in determining live and dead parasites by microscopy after artesunate treatment. The assay was validated by comparing with the original TMI method. To optimize the assay read-out, drug exposure time in LICA was varied from 1, 3 and 5 h. Plasmodium falciparum lactate dehydrogenase activity from parasite lysate was determined to represent parasite viability after 48 h of initial drug treatment instead of using Giemsa staining. The parasite survival measured by using TMI and LICA was compared using Pearson’s correlation.

The early ring stage parasites were adjusted to 1% parasitemia in 2% hematocrit and incubated with artesunate for 3 h. The drug was aspirated and replaced with drug-free complete medium. The plates were incubated for an additional 48 h and kept at ~80 °C. Parasite growth was determined by measuring LDH activity (Gamo et al., 2010). The culture plates were thawed at room temperature and mixed with LDH reaction mixture, consisting of 100 mM sodium L-lactate, 100 μM Tris-HCl pH 8.0. The reaction was conducted at room temperature for 12 min. Lactate was measured using a lactate dehydrogenase assay kit (Cayman Chemical). The percent parasite survival was calculated based on the OD490 of the sample (with drug) compared to the parasite growth control (without drug). The signal was normalized with the background signal (the highest concentration of artesunate). The dose-response curve for IC50 determination was plotted by using GraphPad Prism 6, and Student’s t-test was used for statistical testing.

2.2. Piperaquine and chloroquine sensitivity assays

Piperaquine was kindly provided by WWARN. Chloroquine was purchased from Fluka Analytical (PHR1258). Piperaquine and chloroquine were prepared in 0.5% lactic acid (Sigma) and sterile deionized water, respectively. Synchronous parasites at the ring stage were adjusted to 0.5% parasitemia in 4% hematocrit. The drugs were prepared by two-fold serial drug dilutions in the 96-well plate. The drug concentrations of chloroquine and piperaquine were varied at 500 nM–0.98 nM and 100 nM–0.09 nM, respectively. The plates were incubated at 37 °C for 72 h, and parasite growth after drug treatment was measured using SYBR Green I. Each experiment was done in duplicate for each parasite line. Three biological replicates were performed.

2.3. Artemisinin sensitivity assay

The parasites at the early ring stage (0–6 h post invasion) were prepared by centrifugation at 70% Percoll and subsequently treated with 5% sorbitol (approximately 5 h after Percoll synchronisation). Artesunate was purchased from Guilin Pharmaceutical (Guangxi, China) and was dissolved in 5% sodium bicarbonate. Artesunate was prepared by two-fold serial dilutions in 96-well plate. The drug concentrations were varied (1 μM-1 nM). The IC50 determination for artemesunate was performed by using a newly modified method based on the Trophozoite Maturation Inhibition assay (TMI) (Chotivanich et al., 2014). Lactate dehydrogenase-based Inhibitory Concentration Assay (LICA) was developed in order to reduce the subjective read-out in determining live and dead parasites by microscopy after artesunate treatment. The assay was validated by comparing with the original TMI method. To optimize the assay read-out, drug exposure time in LICA was varied from 1, 3 and 5 h. Plasmodium falciparum lactate dehydrogenase activity from parasite lysate was determined to represent parasite viability after 48 h of initial drug treatment instead of using Giemsa staining. The parasite survival measured by using TMI and LICA was compared using Pearson’s correlation.

The early ring stage parasites were adjusted to 1% parasitemia in 2% hematocrit and incubated with artesunate for 3 h. The drug was aspirated and replaced with drug-free complete medium. The plates were incubated for an additional 48 h and kept at ~80 °C. Parasite growth was determined by measuring LDH activity (Gamo et al., 2010). The culture plates were thawed at room temperature and mixed with LDH reaction mixture, consisting of 100 mM sodium L-lactate, 100 μM 3-acetylpyridine adenine dinucleotide (APAD), 125 μM nitroblue tetrazolium (NBT), 200 μg/ml diaphorase, 0.6% Tween 20, and 35 mM Tris-HCl pH 8.0. The reaction was conducted at room temperature for 30 min in the 96-well half-area clear plate. The signal was determined by optical density at 650 nm (OD650).

Percent parasite survival was calculated based on the LDH signal at OD650 of the sample (with drug) compared to the parasite growth control (without drug). The signal was normalized with the background signal (the highest concentration of artesunate). The dose-response curve for IC50 determination was plotted by using GraphPad Prism 6, and Student’s t-test was used for statistical testing.

3. Results

3.1. Plasmepsin overexpression in P. falciparum

P. falciparum 3D7 parasites were transfected with malarial expression vectors containing plasmepsin II and plasmepsin III with the empty vector as a negative control (designated PM2-3D7, PM3-3D7 and Blank-3D7, respectively). Expression of the respective genes is driven by a P. falciparum calmodulin promoter (Crabb and Cowman, 1996; Wittayacom et al., 2010). The increase in the RNA level was consistently observed using RT-qPCR (Fig. 1). The P. falciparum selectable marker for the episomal expression vector is blasticidin S deaminase (bsd). At 2 μg/ml blasticidin, the level of expression of plasmepsin II and
plasmepsin III transcripts at the trophozoite stages was increased significantly in PM2-3D7 and PM3-3D7 respectively (Fig. 1A). The increases in the transcript levels exceeded the fold changes observed in the field samples (Witkowski et al., 2017). The amount of respective plasmepsin protein was also increased as observed by Western blot analysis (Fig. 1B).

The parasites were tested for their susceptibility to piperaquine and chloroquine. Chloroquine was included in the experiment because of its structural similarity to piperaquine and its clinical use for treating Plasmodium vivax and other non-P. falciparum malaria infections in Southeast Asia. We did not observe a significant change in the levels of piperaquine and chloroquine resistance in the transgenic parasite lines (Fig. 2 and Table 1). Assessment of piperaquine susceptibility was also determined using a piperaquine survival assay (PSA) (Duru et al., 2015). This assay relies on the exposure of the parasites (0–3 h post invasion) to 200 nM piperaquine for 48 h before removing the drug and subsequent monitoring of surviving parasites microscopically after an additional 24 h. Similarly, overexpression of plasmepsin II and plasmepsin III did not increase the number of surviving parasite using the PSA method after piperaquine exposure (Table 1). Recently, a bimodal dose-response curve was proposed to be an alternative readout for piperaquine (α-PM2) and PM3 (α-PM3) with the antibodies against ERD2 (α-ERD2) as a loading control. Signal quantification with Image Studio Lite was normalized with the ERD2 control and presented as fold increase in comparison to Blank-3D7 (shown as numbers under the α-PM3 panel). The fold increase value for PM2 was not available since the PM2 band was not visible in Blank-3D7 even after extended exposure time.

3.2. Effect of plasmepsin overexpression on artemisinin sensitivity

Artemisinin and its derivatives are widely used throughout the tropical world. We wanted to test the effect of plasmepsin overexpression on artemisinin susceptibility. Reduced artemisinin sensitivity was reported in Cambodian patients manifesting as delayed parasite clearance following artemisinin treatment (Ashley et al., 2014; Dondorp et al., 2009). Nevertheless, the level of resistance might not have reached the clinical definition of drug resistance because the drug can still achieve a certain degree of parasite clearance. A shift in in vitro artemisinin susceptibility is also relatively small (Chotivanich et al., 2014). These parasites confer artemisinin resistance during the first 3–6 h of their erythrocytic life cycle. Even though the time window of resistance is relatively small considering the 48-h erythrocytic cycle, it allows a significant portion of circulating ring stage parasites to withstand a pulse of labile artemisinin (Woodrow et al., 2005). This leads to the development of the Ring Survival Assay protocol (RSA) to assess artemisinin sensitivity (Witkowski et al., 2013). In RSA, the early ring parasites are exposed to a pulse of artemisinin for only 6 h, and their survival is determined after 66 h in the absence of artemisinin to determine “Percent Survival”. This method has become a useful tool in tracking these parasites, but the Percent Survival readout does not provide a definite value of artemisinin sensitivity. An alternative method called the Trophozoite Maturation Inhibition assay (TMI) measures the ability of early ring parasites to mature into trophozoites under exposure to different artemisinin concentrations and provides IC50 values (Chotivanich et al., 2014). However, it is demanding as it relies on tedious microscopic visualisation to assess the parasite development at every drug concentration. A robust and simple method for determining artemisinin sensitivity was developed for this study based on colourimetric measurement of P. falciparum lactate dehydrogenase, called the Lactate dehydrogenase-based Inhibitory Concentration Assay.
Drug susceptibility level in the parasites over-expressing plasmepsin II and plasmepsin III.

| Parasite line | Piperaquine | Artesunate | Chloroquine |
|---------------|-------------|------------|-------------|
|                | IC50 (nM) ± SD | p-value | IC90 (nM) ± SD | p-value | IC50 (nM) ± SD | p-value | IC90 (nM) ± SD | p-value |
| PM2-3D7        | 6.86 ± 0.16 0.7716 |          | 9.48 ± 0.97 0.8958 |          | 13.94 ± 1.3 0.3136 |          | 22.49 ± 3.67 0.9739 |          |
| PM3-3D7        | 7.97 ± 0.18 0.49 |          | 11.29 ± 0.15 0.49 |          | 16.49 ± 0.11 0.6 |          | 21.04 ± 2.47 0.59 |          |

PSA survival rate (%) = (Number of viable parasites in exposed culture/Number of viable parasites in non-exposed culture) x 100. The PSA value above ≥10% is considered piperaquine resistance (Duru et al., 2015).

4. Discussion

Here we present the evidence that the overexpression of plasmepsin II and plasmepsin III alone does not affect P. falciparum susceptibility to artesunate, chloroquine or piperaquine. Parasites from Cambodia have developed resistance to piperaquine, a key partner drug to artemisinin. (Amaratunga et al., 2016; Chaorattanakawee et al., 2016). Copy number polymorphism at the genes encoding Plasmepsin II and Plasmepsin III is associated with piperaquine resistance (Amato et al., 2017; Witkowski et al., 2017). This led to the use of their copy numbers as a molecular marker for piperaquine resistance (Imwong et al., 2017). Our findings suggest that gene amplification of plasmepsin II and III is not the causal mutation responsible for piperaquine resistance. Thus, it is important to note that the parasitic field to consider other possible causes underlying piperaquine resistance.

One of the leading candidates is mutations at the gene encoding Plasmodium falciparum chloroquine resistance transporter (PfCRT) (Wellem and Plowe, 2001). The K76T mutation was originally mapped and functionally proven to be a causal mutation for chloroquine resistance (Fidock et al., 2000). Chloroquine and piperaquine share the same 4-aminoquinoline scaffold. It is reasonable to expect that pfcrt mutations could affect parasite sensitivity to piperaquine. In fact, a F145I mutation in PfCRT was shown to be associated with piperaquine resistance especially in the background with plasmepsin copy number polymorphism (Agrawal et al., 2017). Furthermore, a C101F PfCRT mutation was identified during piperaquine selection (Dhingra et al., 2017). This mutation, when introduced into a P. falciparum Dd2 background, significantly increases IC50 values to piperaquine by more than two orders of magnitude (Dhingra et al., 2017). A recent study identified additional PfCRT mutations that have increased in prevalence in Cambodian parasites and confer reduced piperaquine susceptibility when introduced into Dd2 parasites by genome editing (Ross et al., 2018). The reduction in piperaquine susceptibility in transgenic experiments and the GWAS data strongly support the role of PfCRT variants in piperaquine resistance.

It is undeniable that the parasites with plasmepsin copy number polymorphism are spreading in Cambodia (Amato et al., 2018; Imwong et al., 2017). This level of selective pressure could imply the vital role of plasmpenesin gene amplification in providing certain benefits to the parasites. The data presented here provide an experimental proof that introducing extra amounts of Plasmepsin II and Plasmepsin III does not reduce piperaquine susceptibility by itself in the 3D7 parasite genetic (LICA). This LICA method allows a rapid measurement of artemisinin IC50 in P. falciparum. Since artemisinin resistance is manifested only in the early ring stage, 0-6 h-post-invasion parasites were used for subsequent drug-sensitivity assays. Malaria cultures containing 1% early ring parasites were maintained in 96-well plates with a range of artemisinin concentrations from 0.5 nM to 1 μM. Drug-containing medium was removed after a 3-h exposure, which maximized the difference between sensitive and resistant parasites (Fig. 3A). The detail of the parasites and their genetic compositions are previously described (Bunditvorapoom et al., 2018; Ponsuwanna et al., 2016). ANL2 (kelch 13 wild-type) and ANL4 (kelch 13 C580Y) came from the clinical cases with delayed clearance following artemisinin treatment (Bunditvorapoom et al., 2018). To differentiate between signal output from dead and living parasites, the culture was continued in drug-free medium for another intra-erythrocytic asexual cycle to increase the numbers of live parasites since only the surviving parasites can propagate further. P. falciparum lactate dehydrogenase was used as a colourimetric readout. IC50 values using LICA and TMI assays were comparable (Fig. 3B) and were also consistent with the data from the previously published values using the TMI method (Chotivanich et al., 2014). We then tested the effect of plasmepsin overexpression on artemisinin susceptibility. There was no significant shift in their susceptibility to artesunate (Fig. 3C and Table 1).
background. Based on the IC50 data, it also does not affect chloroquine and artesunate susceptibilities. It is possible that plasmepsin II/plasmepsin III gene copy number polymorphism could help with fitness compensation in drug resistance evolution, similar to gdh1 amplification in pyrvinium resistance (Kumpornsin et al., 2014a, 2014b). Further studies on the effect of plasmepsin copy number polymorphism in various genetic backgrounds are necessary because genetic interactions between plasmepsin and other genetic variants might be the underlying driver of piperquine resistance selection.

The use of molecular markers in drug resistance surveillance is an essential component of any malaria control programme. However, it is prudent to weigh the scientific significance of each molecular tool. The role of each malarial drug resistance marker should be exhaustively validated, and any adoption of a drug policy change based on poorly defined molecular markers should be avoided at all cost.

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Appendix A. Supplementary data

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