Author’s response to reviews

Title: Differential proteomic analysis of virus-enriched fractions obtained from plasma pools of patients with dengue fever or severe dengue

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Answers to the Rewievers ( manuscript # INFD-D-15-00240)

Reviewer #1: This study aims to elucidate the proteome associated with dengue viruses from patients with dengue disease (severe or fever). The authors use mass spec techniques on virus-enriched fractions to analyse dengue virions, and follow this up by ELISA. Mass spec analysis yields of dengue-virus containing fractions yielded interesting results, mainly that proteins of the complement system, coagulation system and the acute response signalling are found in fractions with virus. Could the ELISA studies be improved by immunoprecipitation of virus particles (from possibly larger serum volumes) before down stream analysis? The absence of success to confirm viroproteome results by ELISA is perhaps a technical issue that could be worked on. Nonetheless there is useful information in this manuscript as clearly there is an interesting question on why some proteins are enriched at significant difference between two types of patients in the respective virus-containing fractions, and this is of interest to the field. The authors put the data in context by carrying out detailed pathway analysis using IPA. The use of these molecules as prognostic markers would require a more detailed follow up of patients (especially as there appear to be some differences between Colombian and Vietnamese patients) but that is beyond the scope of the paper.
I agree with the reviewer's comments: it would be interesting to confirm that the proteins are indeed associated (or not) with the viral particles. We tried different approaches such as electron microscopy, ELISAs on purified plasmas or on captured virus particles (see lines 425-438). The immunoprecipitation would be another interesting method to try. Unfortunately, the initial plasma volume for each patient is limited (500µL-1mL/patient), large volumes have already been used by previous experiments and for a lot of patients no more sample or few volume are available.

As mentioned in the manuscript (see lines 609-615; 667-668), it seems that the method is more a method to obtain a fraction of a complex biological sample enriched with viral particles than a method that allows to analyze the viroproteome from plasma specimen. The purification method is suitable to MS analysis, because ELISAs have validated two proteins (OLFM4 & PF4) as potential markers of the severity on two different cohorts (see fig.3 and fig. 4).

Specific points:

Introduction: other studies have looked at profiles of immune response/other markers in the serum of patients and while the introduction touches on this, it would be good for the reader to already have this introduced in more detail here.

Many research groups are attempting to assess potential biomarkers for associations with dengue disease progression, and only a selected overview is possible. Predicting outcome in dengue remains challenging, and the search for robust markers/methods is still relevant. Today, the current evidence for any particular clinical or laboratory marker is weak. Meta-analysis of the data is difficult as most of the studies are small, and confounded by serotype variations, differences in the age and immune status of the patients, the timing of sampling during the evolution of the infection etc..

In the first version of the manuscript we discussed this point and gave several references (see “Discussion”, lines 503-507 and ref. 29-34). The potential interest of the coagulation proteins or the Complement factors that correlate with dengue severity was discussed (see lines 564-566; 582-593). In the new version of the manuscript, we have completed the introduction (“Background”) by a paragraph that mentions circulating cytokines/chemokines and endothelial biomarkers as early potential markers of severe dengue (see lines 89-98; ref. 13-15).

Line 235: "non severe dengues" is not an adequate expression in English. I did spot similar points elsewhere in the manuscript and perhaps it should be re-read by a native speaker.

“Non severe dengue” was replaced by “classic dengue” (lines 234 and 413). In addition, the manuscript has been re-read and corrected by two native English scientists.

Methods: a more detailed description of statistics used and power calculations should be provided.
As requested, details on Mann-Whitney and Chi-square tests were added to the “Methods” section (see lines 256-262; ref. 26). In addition, details on Receiver Operating Characteristic (ROC) curves /Area under the ROC curve (AUC) have been added at the end of the “Results” section (see lines 485-490).

Reviewer #2: Manuscript "Differential proteomic analysis of virus-enriched fractions obtained from plasma pools of patients with dengue fever or severe dengue" by Romain Fragnoud and co-authors describe the work done for identification of host proteins co-purified with Dengue virus virions and association of these factors with severity of disease. Two proteins, OLFM4 and PF4, which have prognostic values, have been identified. Such data is interesting and may have practical value.

To my opinion two halves of the study - virion purification/proteomics and subsequent association of identified proteins with disease are rather loosely connected. Authors are aware of this (lines 550-555) and have correctly highlighted that there is no conclusive evidence that the OLFM4 or PF4 are indeed associated with virions. Paper still reads as two separate stories, one dedicated to development and use of new approach to analyze virion proteins and another to association of some markers (not really shown to associate with virions) with severity of disease. However, I agree that presenting these things together is reasonable, as it shows the way the research was planned and performed.

One can also rise a question about biological relevance of the first part of study. The method is not sensitive enough to detect major virus proteins form patient samples (only peptide for E protein was detected, line 305. No peptides from M, Pr of C were revealed. In virus particles these proteins are likely lot more abundant than host proteins. If these major components of virus are detected how sure one can be about identification of less abundant (and possibly loosely associated) host proteins?

Even if the method was initially designed for virus purification and enrichment from complex biological samples, the method finally presented here is a method designed to obtain a fraction of the plasma, depleted of the most represented proteins, enriched with viral particles, and suitable for MS analysis (see lines 609-615; 667-668). As remarked by the reviewer, we have provided no evidence that the identified proteins are indeed associated to the virus. However, for some proteins identified as potential markers, the ELISAs showed that there was a concentration difference between acute plasma specimens from DF and from SD. Consequently, the identification of the markers is probably linked to the use of our purification method. This method allows to reach the deep proteome of plasma that reflects the protein concentration differences between the DF and SD pools.

There are no proof that PF4 and OLFM4 are associated to the virus but ELISA repeatedly confirmed that these markers are potential good markers for the dengue severity prognosis (see fig 3 and 4).

Comments:
1. Real-time RT-PCR has been named/abbreviated differently at different places (lines 116-124, 127). If it is one and the same method it is better to be consistent. As it corresponds to the same method, we wrote “qRT-PCR” for real-time quantitative RT-PCR.

2. Line 229: why were samples pooled before virion purification? Can authors provide rational for this? The samples were both from DEN2 and DEN3 patients, can this possibly reduce specificity? In subsequent analysis also DEN1 patients were included (all Cambodian group was DEN1 infected). This is somewhat confusing

The idea was first to identify a list of markers on pooled samples and then to validate these markers by ELISAs on separate (not pooled) samples.

The samples are pooled to obtain 5mL of plasma, that represents the volume required by our method for virus purification (see “Methods”, line 193). Indeed, for each patient, only 500µL-1mL of plasma are available. By purifying a large volume of sample we would hope to obtain a large quantity of purified virus. In addition, this is the way to analyze 7-8 patient plasmas in one unique experiment.

Ideally, a suitable SD prognosis markers should be present in all the patients of the SD pool, whatever the serotype (and consequently with a high number of peptides identified by MS). At the opposite, a bad prognosis marker should be present in only few patients of the SD pool and thus is “diluted” by the lack of this marker in other patients of the pool (thus with a lower number of peptides identified by MS).

We thought that working on plasma pools both allowed to analyze a lot of samples in one experiment and to screen the markers the most representative for each kind of patients.

The marker concentration may vary according to genetic background, age, presence of co-infections, nutritional status, pre-existing immunity against dengue virus or other flaviviruses, or other environmental factors (see lines 654-661). The serotype can also be a source of variation. Consequently, we constituted the pools with a mix of serotypes. The Colombian patients used for pools constitution are mainly serotype 2 and 3.

The ELISAs are the tools chosen to validate the proteins identified by MS on individual plasma specimens. These makers were first screened by ELISA on 30 Colombian plasmas (serotypes 1, 2 and 3) and then confirmed on 49 Cambodian plasmas (serotype 1) (Table 1 & new Table 3).

3. Line 271, also FIG 1C. Authors provide EM image of particles from mid-stage of purification process. Why do not they show final preparation - the one used directly for proteomics (after Viraffinity polymer purification). It is still possible that final treatment damages particles, so the image of these particles would be more relevant.

We agree with the remark of the reviewer. However, to elute the virus captured by Viraffinity it is necessary to add buffer containing SDS (ie. a detergent) and to heat the polymer few minutes
at 70°C (see Methods, lines 210). By using these conditions it is not possible to see any intact viral particle in the final preparation. That is the reason why we only showed electron microscopy pictures after the first step of the purification (=ultracentrifugations).

4. It would also be interesting to discuss why other two markers (CP1 and C1R) behaved differently between Columbia and Cambodian patients. Could it also be due to the difference of Dengue genotype? There is also interesting difference in viral loads. In Columbia group (table 3) the viral load is higher in SD patients (about 10-fold in average).

It is completely different in Cambodian group (Table 4) where DS patients have in average 3.5 fold lower viral loads. Could this effect the results? Is there any explanation why DF patients in Cambodia have nearly 150-fold higher viral loads? How could this affect the results?

The differences observed between the two cohorts (Colombia and Cambodia) can be due to numerous parameters: serotypes but also genetic background, nutritional status, previous infection by another serotypes…(see lines 654-661). The SD are generally considered more severe in South-East Asia than in Latin America. The age of the SD patients is also lower in Asia (baby-young children). The literature generally associate higher viremia with severity symptoms (see “Background”, lines 119-120). But this point has to be modulated: it has been demonstrated that patients infected with serotype 1 sometimes displayed high viremia levels with a relatively mild clinical disease (Duyen HT, et al. J Infect Dis. 2011;203:1292–1300).

The SD pool used in our MS experiment has a higher viral load compared to the DF pool (see Table 1). The Colombian SD samples used for the ELISAs have a higher average viral load than those of the DF plasmas. For the Cambodian samples, the viremia difference between DF and SD was considered as not significant (see Table 3). The impact of viremia on the marker concentrations seems to be limited and tends to confirm that the method is more a method to reach the deep proteome of the plasma including viral particles (essential for MS analysis) than a method to obtain high purified viral particles (see lines 508-513, 668-671).

5. Line 848. KDa is molecular mass (not molecular weight) unit

Molecular weight has been replaced by molecular mass, as requested.

6. Author could combine tables 3 and 4 - it will easier to see similarities and differences between patients groups. Please also use same units in both tables (even if they are separate tables). Currently total glycerol in table 3 is given in g/L while in table 4 it is mmol/L

As suggested by the Reviewer, the tables 3 and 4 have been combined (>new Table 3).

The total cholesterol units vary according to the origin of the samples and the data available on patient folders. For the Cambodian samples mmol/L has been converted into g/L in the new table 3.