Author's response to reviews

Title: Transforming growth factor beta receptor 1 is a new candidate prognostic biomarker after acute myocardial infarction

Authors:

Yvan Devaux (yvan.devaux@crp-sante.lu)
Melanie Bousquenaud (melanie.bousquenaud@crp-sante.lu)
Sophie Rodius (sophie.rodius@crp-sante.lu)
Pierre-Yves Marie (py.marie@chu-nancy.fr)
Fatiha Maskali (f.maskali@genclis.com)
Lu Zhang (lu.zhang@crp-sante.lu)
Francisco Azuaje (francisco.azuaje@crp-sante.lu)
Daniel R Wagner (wagner.daniel@chl.lu)

Version: 2 Date: 14 November 2011

Author's response to reviews:

Reviewer: Chrishan Samuel

The authors thank Dr Samuel for his evaluation of their manuscript.

This paper utilised blood samples from patients with acute MI, a rat model fo MI, microarrays, qPCR and ELISA kits to demonstrate that TGFBR1 is a potentially novel prognostic biomarker for acute myocardial infarction in humans and rats. Furthermore, the increase in TGFBR1 expression correlated well to known biomarkers of myocardial injury and/or improved the prediction of adverse outcomes. Although this study is of interest and the conclusions drawn fairly well supported by the findings obtained, there are a few queries the authors should address and/or clarify, as detailed below:

Major Compulsory Revisions:
1. The authors ‘introduce’ this study (on page 3) by saying that “A rapid and accurate prediction of the development of HF after MI would be a major breakthrough.”

-However, only one-time point is investigated from both human blood cells and rats post-MI; which appears to be a limitation of this study. Understandably, it would be difficult to study additional time points in humans, but why was only 2-months post-MI chosen as a time point of study in rats? This is a time point of chronic MI, where limited angiogenesis would be occurring in the border region of
the LV.....the authors should additionally demonstrate what happens to TGFBR1 and TGFB1 at earlier time points (ie 2-, 4- and 6-weeks post-MI; when angiogenesis would clearly be expected to occur) or at the very least, better explain why this one time-point was chosen for the experimental studies included.

The authors agree that the study is limited by the absence of a time-dependent evaluation of TGFBR1 expression after myocardial infarction. All human blood samples were collected at presentation. This limitation has been acknowledged in the discussion section page 14 lines 22-25.

We have performed additional experiments in rats. 3 rats were sacrificed 2 days after coronary ligation and 3 rats were sacrificed 2 weeks after coronary ligation. We have observed a significant up-regulation of TGFBR1 staining in the border zone after 2 weeks, not after 2 days. The results are presented in a new panel to figure 4 (panel F) and in the results section page 10 lines 5-6. Of note, we have also measured TGFB1 in tissue homogenates but we did not observe a reproducible tendency, most probably due to the low number of rats investigated.

In rat studies, the 2-months time point was chosen to allow investigation of LV remodeling. With the additional rats sacrificed at earlier time-points, we are now able to show a significant up-regulation of TGFBR1 expression in the heart 2 weeks after coronary ligation.

2. In Table 1, 81-85% of the acute heart failure patients included (in the test and validation cohorts) were men - were the limited female patients included sufficient enough to demonstrate whether gender was a contributing factor to TGFBR1 being a good prognostic marker of acute MI?

23 females were included in the study, 6 in the test cohort and 17 in the validation cohort. This low number is insufficient to allow investigating whether gender contributes to the prognostic value of TGFBR1. In addition, the observation that TGFBR1 expression was not different between women and men (0.32 ± 0.17 vs 0.26 ± 0.14, P=0.25) suggests that gender may not influence the prognostic value of TGFBR1. A paragraph has been added in the discussion section page 13 lines 8-11.

3. In Table 2, the TGFBR1, TGFBR2 and CLU genes were consistently found in all subsets of angiogenic genes associated with LV function. The authors report that the TGFBR2 gene was not detected in their microarrays – but what about the CLU gene? In the logistic regression models used, how did the CLU gene alone or in combination with TGFBR1 classify patients with low/high EF (compared to the combinations outlined in Table 3)?

The CLU gene was detectable on microarrays but very weakly expressed. It is correct that this gene was consistently found in groups of angiogenic genes
associated with LV function in the test cohort. However, we did not measure CLU expression in the validation cohort because the integrated approach used in this study (expression data, networks of protein-protein interaction, prognostic performances) led to the identification of a group of 3 genes (TGFBR1, PTK2 and ITK) providing a maximal AUC of 0.89. All other combinations of genes generated lower prognostic values.

Nonetheless, using microarray data from the test cohort, we observed that CLU classified patients into low or high EF group with an AUC of 0.71. Addition of TGFBR1 did not improve this value. This AUC suggests that CLU may indeed have some prognostic value that should be further evaluated in the validation cohort. Since these analyses are beyond the scope of the present study, we did not include these data in the manuscript, but we thank the reviewer for his thoughtful suggestion.

4. On page 7, the authors state that the 3 genes that best classified patients with low/high EF were the TGFBR1, PTK2 and ITK genes – yet only show data for the TGFBR1 gene. A comparison of how better the TGFBR1 gene was correlated to LV function, compared to the other two genes should be included.

We focused our analyses on TGFBR1 because this gene was the most robustly associated with LV function. PTK2 and ITK did not correlate with the EF ($r=-0.12$, $P=0.20$ and $r=0.19$, $P=0.05$, respectively). These data have been mentioned as “data not shown” page 7 line 17-18. In addition, multiple logistic regression analyses attested that TGFBR1 was the only significant predictor of LV function ($P=0.003$).

5. Do the authors have access to cardiac biopsy tissue from the patients studied – to correlate their findings from blood cells to – to further validate their findings?

It would indeed be interesting to investigate the TGFB1-TGFBR1 axis directly in cardiac tissue, but we unfortunately do not have access to biopsies.

Minor Essential Revisions:

6. The authors should include the coefficient of intrassay (and interassay if appropriate) for the TGF-beta1 ELISA.

Coefficient of variation intra-assay is $<4\%$ and coefficient inter-assay is $<9\%$. This information has been added page 20 line 2.

7. On page 5, the authors should define GSEA before using it as an abbreviation; and on page 15, they should add “...Gene Set Enrichment Analysis (GSEA) software.”

These details have been added.

Reviewer: Timothy McCaffrey
Reviewer's report:

The authors thank this reviewer for his thorough evaluation of their manuscript.

Overall: This is an interesting and potentially important transcriptomic analysis of patients that presented with MI and then went on to maintain, or to lose LV function. The potential predictive value of TGFBR1 in whole blood is potentially important to prognosis, as well as to understanding the LV remodeling process.

Major compulsory revisions:
1) The method of blood collection and profiling needs to be more clearly specified. Was the blood collected before or after mechanical reperfusion? Via arterial line? It was collected into Paxgene tubes?

Blood samples used for TGFBR1 determination were collected into PAXgene tubes, 5 minutes after mechanical reperfusion, and via an arterial catheter. Plasma samples obtained from citrated tubes collected during the same blood puncture were used for TGFB1 measurement. This information has been added to page 16 lines 9-12.

2) Likewise, the array analysis needs details. What kind of array? How was the RNA purified and labeled/amplified? The 1.3 fold change had no statistical confidence, ie t-test?

We initially chose not to overwhelm the methods section with previously published details on microarray analysis (reference 7). The following details have been added page 17 lines 1-18.

RNA was purified on column during the extraction with the PAXgene™ blood RNA kit, and a second purification and concentration step was performed with the RNAeasy® MinElute™ kit. RNA quantity was measured using the ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, USA). RNA quality was assessed using the 2100 Bioanalyzer® apparatus (Agilent Technologies, Massy, France) with the RNA 6000 Nano chips. Only high quality RNA (OD260/OD280 > 1.9 and OD260/OD230 > 1.7) and un-degraded RNA was considered for further analysis. Messenger RNAs were amplified using the Amino Allyl MessageAmp™ kit (Ambion®, Cambridgeshire, United Kingdom) according to the manufacturer's protocol, starting with one µg of total RNA. Five µg of each amino allyl aRNA were labeled with Cy3 or Cy5 (Amersham, Buckinghamshire, United Kingdom). Dye coupling to RNA was measured using the ND-1000 NanoDrop® spectrophotometer. Coupling yield >5% was a prerequisite for further analysis. 750 ng of each amino allyl aRNA labeled Cy3 or Cy5 (reference RNA or patient RNA) were combined and hybridized on oligonucleotide microarrays representing 25,000 genes [14].

We used a threshold of 1.3-fold change to select 525 genes differentially expressed between patients with low EF and patients with high EF. This
selection was not associated with a specific statistical test, as the SAM software. A post-hoc analysis with this software revealed that 54% of the 525 genes were statistically significantly differentially expressed (at q-value <5%). This detail has been added page 5 lines 17-18.

3) Demographic features were similar between groups, except infarct size. So was TGFBR1 predicting or somehow responsive to infarct size?

In the test cohort, patients of the low EF group had larger infarcts than patients of the high EF group. This is expected considering that large infarcts are more prone to induce LV remodeling and dysfunction. We investigated the association between TGFBR1 and infarct size in the validation cohort, which contains a higher number of patients than the test cohort, and which thus allows for more accurate association analyses. By linear regression, both CPK and troponin T predicted TGFBR1 (P=0.03 and P=0.003, respectively), suggesting that TGFBR1 expression is indeed responsive to infarct size. This information has been added page 7 lines 21-24.

4) There needs to be some words of caution around the angiogenesis ‘filtering’ of the data. On the one hand, pathway-based analysis can somewhat minimize the multiple testing problem, but, on the other hand, it takes a method such as microarrays, which derive much of their strength from their broad, hypothesis/bias-free quantitation, and then immediately re-imposes an overt bias for a particular theory: angiogenesis. While angiogenesis is certainly important to the development of collateral circulation, there are many other legitimate pathways that would affect LV remodeling: fibrosis, inflammation, and thrombosis, to name just a few.

The authors fully agree with this comment. Angiogenesis is certainly not the only regulator of LV remodeling, and we have removed the statement that angiogenesis is “a major process” in LV remodeling in the discussion section page 11 line 17.

In the present study, we focused on angiogenesis because previous results from our lab provided evidence that angiogenic genes measured in blood cells from patients with acute myocardial infarction had a prognostic value (Devaux et al. Funct Integr Genomics 2010, 10:329-337.)

LV remodeling is indeed regulated by several other pathways, such as inflammation. Using a systems-level characterization of inflammatory proteins known to be associated with prognosis after acute myocardial infarction, we recently identified a new panel of biomarkers with discriminatory potential (Azuaje et al. BMC Med Genomics 2011, 4:59). Such type of investigations could be extended to other pathways involved in prognosis after myocardial infarction (fibrosis, thrombosis …). A more global approach would be to widen the search for biomarkers to all known pathways that affect LV remodeling, and not to restrain to specific pathways. This latter option could be the aim of future studies,
and has been mentioned page 11 line 25 and page 12 lines 1-2.

5) The angiogenesis theory is also relatively thin even on experimental grounds. If we argue that the arrays on blood are picking up TGFBR1 levels in endothelial progenitor cells (EPC) circulating in blood, their frequency in blood is vanishingly small and unlikely to produce a usable signal. It is more plausible, a priori, that TGFBR1 levels in leucocytes is related to inflammatory activity, or in monocyte-derived macrophages it might predict wound repair functions. These alternate theories deserve mention.

Endothelial progenitors are indeed certainly not the main source of TGBFR1 signal measured by microarrays, considering their extremely low frequency in the circulation, even after acute myocardial infarction. It is correct that circulating monocytes-macrophages may most probably account for the majority of TGFBR1 expression. Interestingly, these cells produce many angiogenic, pro-fibrotic factors (…) which regulate LV remodeling and cardiac healing. The discussion section has been amended accordingly page 14 lines 1-7.

6) While whole blood profile has certain clear advantages, such as avoiding ex vivo artifacts, it leaves open the possible confound of changing populations of cells within blood. Are there CBCs to determine whether there are shifts in cell populations in the 2 groups?

Whole blood cells profiling can indeed be affected by changes in blood cell counts. Following the answer to the previous question, the possibility that the enhanced TGFBR1 expression in patients with low EF is consecutive to increased number of circulating monocytes remains. To address this possibility, we gathered white blood cells counts as well as the percentages of neutrophils, lymphocytes and monocytes for each patient of the test cohort. Patients with low EF had higher WBC counts (15,000 cells/µL for low EF vs 10,000 cells/µL for high EF, P=0.006), higher percentage of neutrophils (84% vs 75%, P=0.01), lower percentages of lymphocytes (8% vs 17%, P=0.001), and comparable percentages of monocytes (4.1% vs 4.5%, P=0.19). Therefore, there is a shift in cell populations between patients with low EF and patients with high EF, attesting for a more robust activation of inflammation in patients with poor prognosis. This confounding factor has been discussed page 14 lines 7-12.

7) Given that the authors speculate as to the potential therapeutic potential, it would be reasonable to inform the reader about potential modes by which the TGFBR1 could be clinically modulated: existing small molecule inhibitors, siRNA, or neutralizing antibodies/ligands for TGF-B.

Therapeutic blockade of the TGFB1-TGFBR1 axis has proven to be effective in pathologies associated with fibrosis. More specifically, inhibition of TGFBR1 activity by orally active specific inhibitors (SD-208 or GW788388 for instance) or
competitive inhibition of TGFB1 by an adenovirus encoding the soluble form of
TGFB2 have been shown to dampen cardiac remodeling after MI. Other
strategies to block TGFB, which have been recently reviewed by Hawinkels and
Ten Dijke. (Growth Factors 2011;29:140), may also be tested to limit cardiac
remodeling. The discussion section page 13 lines 21-25 has been reformulated
accordingly.

Minor compulsory revisions:
8) The third person narrative would be preferred: “Patients with MI were
enrolled…” as opposed to “We enrolled patients with MI…”

This has been changed page 5 line 4.

9) This sentence is confusing. Is it meant to say that these germline diseases
suggest that blocking the receptor systemically would have adverse outcomes, or
that you would not be able to use this as a diagnostic in people with germline
defects? “In addition, mutations in
TGFB1 gene, known to affect vascular integrity in Marfan and Loeys-Dietz
syndromes [34, 35], may limit the use of TGBR1 as either a marker or a
therapeutic
target after acute MI.

The authors believe that mutations in TGFBR1 may affect, to some extent, both
the diagnostic and therapeutic use of TGFBR1. First, sequence alterations may
decrease the specificity of probes used to measure TGFBR1 expression, and this
would have to be considered when designing the probes. Second, mutations may
affect the binding of specific inhibitors, either small molecules or neutralizing
antibodies. The affinity of these inhibitors for the mutated form of the receptor
would therefore have to be evaluated. The sentence page 15 lines 1-3 has been
reformulated.

Reviewer: Nikolaos G Frangogiannis
Reviewer’s report:
The study by Devaux and co-workers studies the predictive role of blood cell
TGF-betaR1 (TbetaR1) expression and plasma TGF-beta in patients with acute
myocardial infarction. The authors found that blood cell TbetaR1 was associated
with LV dysfunction 4 months after STEMI; TbetaR1 added some value to the
predictive potential of troponin T.
General comment:
This is an interesting study using a systems biology approach to identify
biomarkers that may predict the development of adverse remodeling following
infarction. Study of the circulating blood cell transcriptome is of interest as it provides information on inflammatory and reparative signals. Identification of TbetaR1 as a biomarker is novel. However, the following major concerns are raised:

The authors thank this reviewer for his critical evaluation of their manuscript.

Major comments:
1) The findings may reflect, for the most part, enhanced activation of TGF-beta/TbetaR1 in patients with larger infarcts, who are known to mount a more intense inflammatory response. Was the predictive value of TbetaR1 independent of the size of the infarct?

In the test cohort, patients of the low EF group had larger infarcts than patients of the high EF group. This is expected considering that large infarcts are more prone to induce LV remodeling and dysfunction. We investigated the association between TGFBR1 and infarct size in the validation cohort, which contains a higher number of patients than the test cohort, and which thus allows for more accurate association analyses. By linear regression, both CPK and troponin T predicted TGFBR1 (P=0.03 and P=0.003, respectively), suggesting that TGFBR1 expression is responsive to infarct size. The AUC of CPK and troponin T was 0.64 for both, and addition of CPK or troponin T did not improve the prognostic value of TGFBR1 (AUC = 0.73 for TGFBR1 and AUC = 0.72 for TGFBR1 and CPK or troponin T). These results argue against a contribution of infarct size in the prognostic value of TGFBR1. This information has been added to page 7 lines 21-24, page 8 lines 5-7 and in Figure 3B.

2) The animal study is weak and provides very limited new information:
   a. TGF-beta1 in the rat myocardium was studied at a single timepoint; because of the dynamic nature of the reparative response there are significant changes in TGF-beta levels as the infarct heals.

   We have performed additional experiments in rats. 3 rats were sacrificed 2 days after coronary ligation and 3 rats were sacrificed 2 weeks after coronary ligation. We have observed a significant up-regulation of TGFBR1 staining in the border zone after 2 weeks, not after 2 days. The results are presented in a new panel to figure 4 (panel F) and in the results section page 10 lines 5-6. Of note, we have also measured TGFB1 in tissue homogenates but we did not observe a reproducible tendency, most probably due to the low number of rats investigated.

   In rat studies, the 2-months time point was chosen to allow investigation of LV remodeling. With the additional rats sacrificed at earlier time-points, we are now able to show a significant up-regulation of TGFBR1 expression in the heart 2 weeks after coronary ligation.

   b. Large amounts of latent TGF-beta is present in the myocardium; the levels
assessed through ELISA in homogenized tissues do not reflect TGF-beta bioactivity in vivo.

The ELISA used to measure TGFβ1 detects only the activated form. In initial analyses, we measured total TGFβ1 since latent TGFβ1 was activated before performing the assay. We have done additional tests without activation of latent TGFβ1 and we have found that the level of activated TGFβ1 in the myocardium 2 months after induction of MI was comparable to sham animals. This observation suggests that the up-regulation of TGFβ1 measured after MI (Figure 4A) reflects an increase of latent TGFβ1.

This point has been clarified in the Methods section (page 19 line 25 and page 20 line 1), in the Results section (page 9 lines 18-22), and in the legend to Figure 4A (page 27 line 14).

c. Immunohistochemical analysis of TβR1 expression does not identify the cells expressing the receptor. In any case, the receptor is present in all cells, but may be upregulated in reparative cells infiltrating the infarct.

The authors should recognize the many limitations of their animal study.

Our starting observation was the enhanced expression of TGFβR1 in circulating leukocytes of patients with acute myocardial infarction. We recognize that our animal study would deserve a much deeper analysis, such as determining which cell type(s) express and regulate TGFβR1 in the infarcted heart. This was however not the main goal of the present study, which focuses on the prognostic value of TGFβR1 in humans. We have added a paragraph to the discussion section (page 14 lines 12-16).

3) The findings would better reflect post-infarction remodeling had the authors excluded patients with a previous MI or underlying cardiomyopathy.

Five patients of the test cohort (16%) had a previous MI. In the validation cohort, 7 patients (6%) had a previous MI. In the 108 patients of the validation cohort who presented with a first MI, the predictive value of TGFβR1 had an AUC of 0.72, identical to the AUC reported for the whole validation cohort. Therefore, although we agree that a population of patients with first infarcts would have better reflected post-MI LV remodeling, the presence of few patients with a second infarct did not affect our results. A paragraph has been added to the results section page 8 lines 9-14.

4) Relations between TβR1/TGF-beta and remodeling-associated parameters should be studied and could provide important insights. These parameters could include chamber dimensions and the change in chamber dimensions/function during the remodeling period. Because the TGF-beta axis is also associated with cardiac hypertrophy relations with LV mass would be relevant.

Dissection of the mechanisms by which activation of the TGFβR1-TGFβ1 axis
regulates LV remodeling would indeed greatly benefit from the investigation of the associations with LV parameters. Unfortunately, we do not have data for LV parameters (chamber dimensions, change between presentation and follow-up, LV mass) for the patients used in this study. Also, chamber dimensions and LV mass were not determined in the animal study.

We have measured cardiomyocytes cross-sectional area on tissue sections, and we did not find any significant correlation with TGFB1. The association between TGFB1 and LV hypertrophy is evidenced by the positive correlation between TGFB1 and the change in LV volumes (Figure 4C-D).

5) The abstract needs to be revised to clarify important issues: a. “blood samples” does not accurately define the samples obtained; please indicate that mRNA extracted from whole blood cells was studied. b. Please define the patient population as individuals with STEMI who underwent primary PCI.

These changes have been performed.

Minor comments:
Please correct typos in reference 19, please update reference 27 (indicated as in press) and complete reference 43.

References have been corrected.