PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Association of digital vascular function with cardiovascular risk factors: a population study
AUTHORS	Kuznetsova, Tatiana; Van Vlierberghe, Eline; Knez, Judita; Szczesny, Gregory; Thijs, Lutgarde; Jozeau, Dominique; Balestra, Costantino; D'hooge, Jan; Staessen, Jan

VERSION 1 - REVIEW

REVIEWER	Christian Delles Institute of Cardiovascular & Medical Sciences University of Glasgow Scotland, UK
REVIEW RETURNED	16-Dec-2013

GENERAL COMMENTS	This is an interesting paper looking at a new technique to study digital vascular function in a general population cohort. The authors demonstrate a number of associations between PWA hyperaemic responses and traditional cardiovascular risk factors. Most notably, digital vascular function was associated with sex and BMI. The paper also benefits from an in-depth analysis of the hyperaemic response at different time intervals following cuff pressure release according to a previously described protocol for another device (Endo-PAT2000; Hamburg et al., reference 10). Whilst generally well written and providing interesting data I feel that the authors have not exhausted the potential of this study and could perform additional analyses to generate additional data. 1. The new technique is interesting, but I couldn't find a description of its potential advantages and disadvantages compared to other techniques measuring digital vascular function (Endo-PAT2000) and endothelial function in general. The authors may wish to add a paragraph on this issue. 2. The “response rate of 85.1% is not clear to me. It would be important to describe the selection criteria of the originally invited 444 (?) people. 3. What were the criteria for starting the ischaemic phase? I note that it was started after 2 minutes and 20 seconds, but it would be important to ensure a stable baseline signal before commencing the ischaemic phase. It would also be important to learn how breakthrough signals indicating minimal flow during the ischaemic phase were avoided (there are a few in figure 1D that may affect the quality of ischaemia). 4. The sample of 5 subjects in whom inter-session reproducibility was studied appears very small. Are the authors sure that this will

	produce reliable data on reproducibility especially on those with more extreme PPG PWA results? 5. What I am really missing are data on the baseline PWA signal. The authors exclusively analysed the ischaemic response, but it would be interesting to see the determinants of baseline PWA. One would, for example, expect higher flow in people who are on calcium channel blockers or other vasodilators. Does baseline flow determine the ischeamic response? It could well be that those with higher baseline flow have a reduced capacity to further increase PWA. I would strongly encourage the authors to study baseline PWA as part of this paper. 6. I am surprised to see a prevalence of hypertension of 43.1% in this general population cohort. Is this not slightly higher than expected? The definition of hypertension is also not entirely clear. It appears that in part this was based on BP readings of >140/90 mmHg but also on questionnaire data. Can this be disentangled? It seems that the 43.1% exclusively derive from the questionnaire data [(54+80)/(154+157)] so I am not sure how this relates to the 5 blood pressure measurements and the 140/90 criterion on page 8. 7. The authors did not take advantage of the availability of data from two fingers. It would be interesting to see the correlations of baseline PWA between right and left hand. Surprisingly, the example chosen for figure 1C shows a marked difference in baseline flow between the two sites and I wonder if this was a common observation. 8. The small change in flow in the control arm is mentioned in the results section. How can this be explained and was it generally observed? Again, this requires formal analysis as it may indicate an interesting phenomenon. 9. Are the data on the top of page 10 on cholesterol adjusted for statin use? 10. In general, the present study is an important first step as one would expect a measure of endothelial function to be correlated with CV risk factors. However, in terms of being an independent CV risk predictor one would indeed hope that not all of the variability of this measure is explained by already known risk factors. This should be mentioned somewhere in the discussion. Other issues 1. In the results section of the abstract and also on page 9 of the manuscript, the sentence on “totaled from 9.2% at 0-30 second-interval and 22.5% at ...” is not clear. Does this mean “totaled from xxx% to xxx%”, i.e. describing a range with a minimum and maximum? 2. The first bullet point in “Article focus” of the article summary should be modified. It is not vasodilation “after reactive hyperaemia” but rather “during reactive hyperaemia” (as the authors correctly write in line 12, page 5) or “associated with reactive hyperaemia”. Also, it is a general limitation of techniques assessing digital vascular function that the vascular bed under study (finger) is within the vascular bed that was subject to ischaemia (forearm). Thereby the observed vasodilation is induced by both ischaemia and shear

	stress. This is different from brachial artery FMD. Somewhere in the discussion this limitation should be mentioned. 3. I disagree with the first bullet point in “Key messages” of the article summary. The authors did not demonstrate that PPG PWA is a measure of endothelial function. This is well possible and in fact likely, but no data on an effect of NO synthase inhibition on the signal or comparisons with other methods to assess endothelial function have been provided. 4. First bullet point in “Strengths and limitations of the study” in the article summary. Here and generally in the article I am missing data on other studies using PPG PWA. If this is the first study in a general population cohort one would like the authors summarising what has been done with PPG PWA in disease cohorts so far. 5. Fourth bullet point in this section. This should be rewritten or deleted. “Further prospective” sounds as if the present study was already prospective – this is not true. It is also not clear why prospective studies are required to validate PPG PWA as a measure of endothelial function. This can be done e.g. by studies into the effects of GTN or L-NMMA on the signal. Prospective studies may, however, demonstrate the value of the technique to predict CV outcomes. 6. Page 10, second paragraph, line 2, please say “hyperaemic”; and in the last line of this paragraph “compared”. In the first line of the discussion please say “relationship between...”. 7. Figure 2. One of the beige/olive open circles is filled. Why?

REVIEWER	Tine De Backer University Hospital Ghent Belgium
REVIEW RETURNED	24-Dec-2013

GENERAL COMMENTS	This is an interesting paper given the fact that endothelial function is a domain where many researchers have tried and try to measure endothelial function as good as possible. It is a complex area and examination of endothelial function is tricky. What is endothelial function? What represnets endothelial fucntion? How can endothelial function be accurately measured? etc The release of vasoactive substances differs from small muscular to large muscular to elastic arteries. Some authors even point out that same sized (muscular) arteries can exhibit differences in endothelial response (cfr coronary ae compared to other ae). The authors of this manuscript sought to evaluate the correlates of digital PPG pulse amplitude hyperaemic responses as a measure of peripheral arterial function in a sample of a general population. -How sure are the authors that changes in the pulse amplitude (measured by PPG) represent endothelial function? How sure are they that they are actually measuring endothelial function? The conclusion sentence seems more appropriate in representing the study:" We evaluated the relation of PPG pulse amplitude hyperaemic response, a noninvasive measure of peripheral microcirculation, to cardiovascular risk factors. The authors also correctly state at the end of the conclusion of the manuscrip that further studies are required to validate the PPG technique for the non-invasive assessment of endothelial function. -Would it not have been better if PPG was compared to another valid measure for peripheral arterial function? -"Under strictly controlled conditions" ..Can the authors summarize what these conditions should be? And what about people taking vitamins? medication? food and drinks? -What about influence of temperature? Was skin temperature controlled? Finger temperature? -Asessemnt of independent correlations of the PPG pulse amplitude ratio was done with several factors like total cholesterol: what about LDL? Blood glucose? (viscosity?) -Were PPG responses different in hypertensives versus normotensives? -PPG PWA changes decreased with male sex: was there taken into account that the men in the cohort had higer blood pressures? and some of them had a history of ischemic heart disease? -The hyperaemic response descreased with smoking: was this similar for men and women?

	-The inverse correlation with BMI: (besides indeed the possiblity of the role of metabolic stress factors) could that simply be due to the technique which is based on a light signal sent into tissue? -p11: ... "several minutes" : proposal to replace by "5 minutes" -p11:"compared with the method based on finger applanation tonometry": proposal to write .. "compared with results from studies using the finger applanation tonomtery based method." - Is the sex difference mainly due to allometry? Or could other factors such as hormones play an important role in this? - How could it be explained that there is no effect of antihypertensive or lipid lowering therapy? Was the lipid lowering therapy mainly consisting of statins? - How could it be explained that no change with age is observed? -Given the fact that there is no correlation between brachial and digital measures of vascular function, can one conclude that the two methods do not/cannot examine the same? -How sure can it be stated that the PPG is a usefool in detection endothelial dysfunction? Should comparison with FMD ("gold standard") have been performed? -Concerning the stepwise multiple regression: forwards or backwards? -Were there confounders? -Fig 3 ..;"adjusted for important covariables": which covariables?

VERSION 1 – AUTHOR RESPONSE

Reviewer 1

Question Nr 1: The new technique is interesting, but I couldn't find a description of its potential advantages and disadvantages compared to other techniques measuring digital vascular function (Endo-PAT2000) and endothelial function in general. The authors may wish to add a paragraph on this issue.

The objective of our study was to evaluate the correlates of digital PPG PWA hyperemic responses as a measure of peripheral vascular function. Our study was not designed to formally compare two available techniques for assessment of digital vascular function - tonometry and photoplethysmography. However, as requested by the Reviewer, we added the following sentence (page 12, first paragraph): "Both techniques used to assess digital vascular function are non-operator-dependent, and the equipment is an order of magnitude less expensive than for ultrasonography. However, the tonometry method might be more expensive as compare to the PPG techniques because of additional costs associated with changeable plethysmographic probes. Furthermore, the digital tonometry procedure is more complicated and less comfortable for patients because it requires attachment of a pneumo-electrical tube to an additional pneumatic digital cuff which should be constantly inflated during the test."

Question Nr 2: The “response rate of 85.1% is not clear to me. It would be important to describe the selection criteria of the originally invited 444 (?) people.

As requested by the Reviewer, we clarify the study population as follows (page 6; first paragraph): " The initial participation rate was 78.0%. The FLEMENGHO study is on-going longitudinal population study and, therefore, the participants were repeatedly visited at home and examined at a local examination centre. From January 2010 until March 2012 a scheduled follow-up examination included also measurement of endothelial function with the PPG technique. From 444 invited participants for this examination, we obtained informed written consent from 378 subjects (response rate 85.1%)."

Question Nr 3: What were the criteria for starting the ischaemic phase? I note that it was started after 2 minutes and 20 seconds, but it would be important to ensure a stable baseline signal before commencing the ischaemic phase. It would also be important to learn how breakthrough signals indicating minimal flow during the ischaemic phase were avoided (there are a few in figure 1D that may affect the quality of ischaemia).

As we explained in Methods (see page 6 and 7 of the revised manuscript), to determine the amplitude changes of the digital pulse curve in response to hyperaemia, we used a protocol as described by Hamburg et al (see ref 6). To attain a cardiovascular steady-state before starting the test, the participants had rested for at least 20 minutes in the supine position. After that, as shown in Figure 1, panel C and D, baseline PPG pulse amplitude was registered for at least 5 minutes to ensure a stable baseline PPG signal. According to the protocol (see ref 6), for the analysis, we used PPG pulse amplitude that was measured for last 2 min 20 sec. Next, arterial flow was interrupted for 5 minutes by an inflatable cuff placed on the proximal forearm with an occlusion pressure of 200-220 mmHg (around 50 mmHg above the participant’s systolic pressure). Complete cessation of blood flow to the hand is verified by the absence of a PPG signal from the occluded arm. Although the participants were asked to refrain from moving the figures, as it could create mechanical artefacts, sometimes we observed signals (spikes) from the occluded arm. All these "breakthrough" signals were checked and we confirmed that they did not match the signal from the control arm and, therefore, represented mechanical artefacts.

Question Nr 4: The sample of 5 subjects in whom inter-session reproducibility was studied appears very small. Are the authors sure that this will produce reliable data on reproducibility especially on those with more extreme PPG PWA results?

We agree with the Reviewer that the sample of 5 subjects in whom we performed inter-session reproducibility of a PPG signal might be small if we considered only the peak PPG pulse amplitude ratio. In fact, we also reported the reproducibility of the averaged PPG PWA ratio per each 30-second time interval (we used 7 time intervals in this analysis). As shown in attached Figure 1, in our reproducibility study, we did not select subjects with extreme PPG PWA results. In the revised manuscript we included reproducibility only of the averaged PPG PWA ratios (page 8, first paragraph). The intra- and inter-session reproducibility study on a larger sample of subjects using a beta-version of the PPG device is planned.

Question Nr 5: What I am really missing are data on the baseline PWA signal. The authors exclusively analysed the ischaemic response, but it would be interesting to see the determinants of baseline PWA. One would, for example, expect higher flow in people who are on calcium channel blockers or other vasodilators. Does baseline flow determine the ischeamic response? It could well be that those with higher baseline flow have a reduced capacity to further increase PWA. I would strongly encourage the authors to study baseline PWA as part of this paper.

To address the comment of Reviewer on baseline PPG PWA, we would like to emphasise that the photoplethysmogram represents a circulatory signal related to the pulsatile volume of blood in tissue. It has been already demonstrated in many studies (see review by Warner et al, Anaesthesiology 2008;108:950-8) that the appearance of the PPG signal depends on many optical, biomechanical and physiological covariates. For instance, light intensity is attenuated by oxygenated and deoxygenated haemoglobin and blood glucose, as well as by melatonin (in skin) and other optically significant compounds in bone and connective tissue. Along with these lines, in our study, we found that the absolute baseline PPG PWA amplitude is decreased with age (P=0.01; this might be related to changes in connective tissue and skin properties that we often observe in elderly subjects). As expected, we also observed a direct significant association of the absolute PPG PWA amplitude with pulse pressure (P=0.04) and blood glucose (P=0.026), but not with chronic antihypertensive drug treatment. In our study, we would like to stay focus on relative changes in the PPG PWA signal in response to reactive hyperaemia, rather than on characteristics of absolute values of PPG amplitude which could be affected by many cofactors related to the methods as demonstrated in previously published studies.
As explained in Methods to calculate the PPG PWA ratio we used the baseline PPG amplitude (PAht/PAh0, where PA is the pulse amplitude, h is the hyperaemic finger, t is time interval, and 0 is baseline). Therefore, in our opinion, it could be statistically inappropriate to explore a correlation between the PPG ratio and the baseline PPG amplitude. In this case, one always find a correlation between these variables.
In the revised manuscript we added the following information on the baseline PPG amplitude (page 9, last paragraph): "The geometric means of the baseline PPG amplitude were 7.3 (5%-95% percentiles: 2.7 to 25.9) and 9.3 (5%-95% percentiles: 3.9 to 25.3) at the hyperaemic and control finger, respectively. We observed a high correlation between values of the baseline PPG amplitude recorded at both fingers (r=0.89, P<0.0001)".

Question Nr 6: I am surprised to see a prevalence of hypertension of 43.1% in this general population cohort. Is this not slightly higher than expected? The definition of hypertension is also not entirely clear. It appears that in part this was based on BP readings of >140/90 mmHg but also on questionnaire data. Can this be disentangled? It seems that the 43.1% exclusively derive from the questionnaire data [(54+80)/(154+157)] so I am not sure how this relates to the 5 blood pressure measurements and the 140/90 criterion on page 8.

The prevalence of hypertension reported in this study (43.5%) was in line with what we previously reported (see ref 8). Needless to say, the reported prevalence of hypertension depends on mean age of the studied population (in our case it was 52.6 years). As we explained in Methods, hypertension was a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic (average of 5 consecutive auscultatory readings at the examination centre) or the use of antihypertensive drugs as reported in the questionnaire. To avoid further confusion, we removed the number of hypertension subjects from the questionnaire data section of Table 1.

Question Nr 7: The authors did not take advantage of the availability of data from two fingers. It would be interesting to see the correlations of baseline PWA between right and left hand. Surprisingly, the example chosen for figure 1C shows a marked difference in baseline flow between the two sites and I wonder if this was a common observation.

Please, refer to our replies to your comment 5.

Question Nr 8: The small change in flow in the control arm is mentioned in the results section. How can this be explained and was it generally observed? Again, this requires formal analysis as it may indicate an interesting phenomenon.

According to the standardized protocol and as described elsewhere (see ref 6), we normalized the PPG PWA ratio to measurements from the contra-lateral arm, which serves as control for non-endothelial dependent systemic effects. Most notably, this normalization controls for fluctuations in sympathetic nerve outflow that may induce changes in peripheral arterial tone that are superimposed on the hyperaemic response. We agree with Reviewer that this phenomenon might be interesting to further explore, however, it will require a larger sample size as these fluctuations are minimal (see Figure 2 in the revised manuscript). On the other hand, in our present report we would like to stay focus on correlates of endothelial dependent vasodilatation.

Question Nr 9: Are the data on the top of page 10 on cholesterol adjusted for statin use?

In our stepwise regression analysis lipid-lowering drugs (statins) was not selected as an independent determinant of the PPG pulse amplitude ratio at any time intervals (P-values for variables to enter and stay in the regression model were set at 0.10). However, as requested by Reviewer after additionally adjustment of our regression model for lipid-lowering drugs, the results on cholesterol remained confirmative (P=0.043).

Question Nr 9: In general, the present study is an important first step as one would expect a measure of endothelial function to be correlated with CV risk factors. However, in terms of being an independent CV risk predictor one would indeed hope that not all of the variability of this measure is explained by already known risk factors. This should be mentioned somewhere in the discussion.

As shown in Table 2, in a general population, we could explain around 22% of variability of the peak PPG PWA ratio by traditional CV risk factors, including male sex, current smoking, body mass index and total cholesterol. The remaining variability might be influenced by genetic factors, inflammatory processes or other confounders that we did not consider in our study. Moreover, in our opinion, it is important to demonstrate in prospective studies that the hyperaemic response as assessed by the PPG technique might be an independent predictor of CV events. We modified Discussion accordingly (page 14, first paragraph).

Other issues

10. In the results section of the abstract and also on page 9 of the manuscript, the sentence on “totaled from 9.2% at 0-30 second-interval and 22.5% at ...” is not clear. Does this mean “totaled from xxx% to xxx%”, i.e. describing a range with a minimum and maximum?

We modified this sentence according to suggestion of Reviewer.

11. The first bullet point in “Article focus” of the article summary should be modified. It is not vasodilation “after reactive hyperaemia” but rather “during reactive hyperaemia” (as the authors correctly write in line 12, page 5) or “associated with reactive hyperaemia”. Also, it is a general limitation of techniques assessing digital vascular function that the vascular bed under study (finger) is within the vascular bed that was subject to ischaemia (forearm). Thereby the observed vasodilation is induced by both ischaemia and shear stress. This is different from brachial artery FMD. Somewhere in the discussion this limitation should be mentioned.

We modified the first bullet point in "Article focus" according to suggestion of Reviewer.
We also added the sentence on effect of ischaemia and shear stress on vasodilation in the section on study limitations (page 14, first paragraph).

12. I disagree with the first bullet point in “Key messages” of the article summary. The authors did not demonstrate that PPG PWA is a measure of endothelial function. This is well possible and in fact

likely, but no data on an effect of NO synthase inhibition on the signal or comparisons with other methods to assess endothelial function have been provided.

Theunissen et al (ref 18) observed in divers an increase in circulating NO after successive breath-hold dives. This increase in circulating NO level was associated with higher hyperaemic response measured using the same PPG device as in our study. Because in this study, for the first time, the authors demonstrated the association between changes in NO level and hyperaemic response measured by the PPG method, we included this important reference in Discussion (page 12, first paragraph). In any case, as suggested by both Reviewers (see comment 1 of Reviewer 2), we modified the first bullet point in "Key messages" as follows: "We demonstrated that measurement of the hyperaemic response by the new PPG technique might be a useful tool in the detection of microvascular dysfunction associated with cardiovascular risk factors."

13. First bullet point in “Strengths and limitations of the study” in the article summary. Here and generally in the article I am missing data on other studies using PPG PWA. If this is the first study in a general population cohort one would like the authors summarising what has been done with PPG PWA in disease cohorts so far.

In our study, we analysed the changes in maximal amplitude of the PPG signal during reactive hyperaemia. Apart of the abovementioned study on divers, to our knowledge, there are no other publications using the PPG PWA technique to evaluate the hyperaemic response. In one study Chowienczyk et al (J Am Coll Cardiol 1999) measured point of inflection ("notch", IP) on photoplethysmographically obtained digital pulse wave curve in response to salbutamol and glyceryl trinitrate (GNT) in 20 diabetic patients and 20 controls. In this study, the authors demonstrated that relative to maximal amplitude IP height were blunted in diabetic patients after systemic administration of salbutamol as compare to control subjects.

14. Fourth bullet point in this section. This should be rewritten or deleted. “Further prospective” sounds as if the present study was already prospective – this is not true. It is also not clear why prospective studies are required to validate PPG PWA as a measure of endothelial function. This can be done e.g. by studies into the effects of GTN or L-NMMA on the signal. Prospective studies may, however, demonstrate the value of the technique to predict CV outcomes.

As suggested by Reviewer we changed the fourth bullet in "Strenghts and limitations" as follows: "Further research including clinical and prospective epidemiological studies are required to validate the PPG technique for non-invasive assessment of endothelial function and prediction of cardiovascular outcome, respectively".

15. Page 10, second paragraph, line 2, please say “hyperaemic”; and in the last line of this paragraph “compared”. In the first line of the discussion please say “relationship between...”.

We addressed the minor comments as suggested by Reviewer.
16. Figure 2. One of the beige/olive open circles is filled. Why?
We corrected Figure 2.

Reviewer 2
Question Nr 1: How sure are the authors that changes in the pulse amplitude (measured by PPG) represent endothelial function? How sure are they that they are actually measuring endothelial function? The conclusion sentence seems more appropriate in representing the study:" We evaluated the relation of PPG pulse amplitude hyperaemic response, a noninvasive measure of peripheral microcirculation, to cardiovascular risk factors. The authors also correctly state at the end of the conclusion of the manuscrip that further studies are required to validate the PPG technique for the

non-invasive assessment of endothelial function.

Please, refer to our replies to comment 12 of Reviewer 1.

Question Nr 2: Would it not have been better if PPG was compared to another valid measure for peripheral arterial function?

We agree with Reviewer that direct comparison of different methods in is important. However, the objective of our study was to evaluate the correlates of digital PPG PWA hyperemic responses as a measure of peripheral vascular function. Our study was not designed to formally compare available techniques for assessment of digital vascular function. On the other hand, our results are consistent with those of other studies, in which finger applanation tonometry was used to assess the digital function and, therefore, constitute an internal validation of the PPG techniques in assessment of digital vascular function. As we pointed out in Discussion, so far, all non-invasive methods used to estimate endothelial function including FMD have their potential disadvantages and measure different type of reactivity: microcirculatory vs macrocirculatory.

Question Nr 3-5:"Under strictly controlled conditions" ..Can the authors summarize what these conditions should be?
And what about people taking vitamins ? medication ? food and drinks?
What about influence of temperature? Was skin temperature controlled? Finger temperature ?

As requested by the Reviewer, we further clarify the conditions. We followed the standardized protocol as described in detail in Methods (page 6, last paragraph):
"The participants refrained from smoking, heavy exercise, and drinking alcohol or caffeine-containing beverages for at least 3 hours before the test. No medication was taken on the day of the examination. We studied endothelial function in an air-conditioned room at constant temperature around 22°C. To attain a cardiovascular steady-state before starting the test, the subjects had rested for at least 20 minutes in the supine position. Since peripheral vasoconstriction is correlated with the surrounding temperature, before the test, special care was taken to keep fingertips temperature around 35°C. The blood pressure was the average of 5 auscultatory readings, obtained with a standard sphygmomanometer. The blood pressure measurement was performed on the arm that served as control."

Question Nr 6: Assessment of independent correlations of the PPG pulse amplitude ratio was done with several factors like total cholesterol: what about LDL? Blood glucose? (viscosity?)

The objective of our study was to explore the associations between hyperaemic responses as a measure of peripheral vascular function and established cardiovascular risk factors such as body mass index, smoking, total cholesterol, etc. Our results are consistent with those of other studies, in which finger applanation tonometry was used to assess the digital function and, therefore, constitute an internal validation of the PPG techniques in assessment of digital vascular function. As requested by Reviewer we performed additional stepwise regression analyses and included in our models additionally LDL, haematocrit (as measure of blood viscosity) and fasting blood glucose. P-values for variables to enter and stay in the regression model were set at 0.10. In this additional stepwise regression analysis LDL and haematocrit were not selected as an independent determinant of the PPG pulse amplitude ratio for any intervals. On the contrary, blood glucose was also selected as an independent determinant of the PPG ratio (see Table 2 in the revised manuscript), but overall impact of this covariable is relatively small (explained about 1,5% of total variability). Moreover, blood glucose was not a significant determinant of the maximal peak of hyperaemic response which occurs at 30- to 60 second and 60- to 90-second intervals. We also noticed that blood glucose is associated with the baseline absolute PPG PWA amplitude (P=0.026). This association might be related to the

optic technique we used in our study. Indeed, blood glucose concentration could be measured using the photoplethysmography by obtaining a differential optical density related to the blood component in the finger tissues (Yamakoshi Y et al, Conf Proc IEEE Eng Med Biol Soc. 2009).

Question Nr 7: Were PPG responses different in hypertensives versus normotensives?

In unadjusted and in multivariable adjusted (see attached Figure 2) analyses, we did not observe significant differences in hyperaemic response throughout the 0- to 120-second post deflation intervals (P≥0.20).

Question Nr 8: PPG PWA changes decreased with male sex: was there taken into account that the men in the cohort had higher blood pressures? and some of them had a history of ischemic heart disease?

After additional adjustment for systolic blood pressure and previous history of ischemic heart disease (n=7), the associations between the PPG PWA ratios and male sex remained consistent.

Question Nr 9: The hyperaemic response decreased with smoking: was this similar for men and women?

As suggested by Reviewer, we tested if there is any differences in the hyperaemic response with smoking among women and men. We did not observe significant interaction between sex and smoking in relation to the hyperaemic response (P≥0.57).

Question Nr 10: The inverse correlation with BMI: (besides indeed the possibility of the role of metabolic stress factors) could that simply be due to the technique which is based on a light signal sent into tissue?

As we explained in Discussion, similar to other studies, in which the different technique (finger applanation tonometry) was used to assess the endothelial function, we also observed a significant relation between hyperaemic PPG pulse amplitude changes and body mass index. In our view, these findings constitute an internal validation of the PPG techniques in assessment of digital vascular function which could be used together with applanation tonometry .

Other issues
Question Nr 11: Is the sex difference mainly due to allometry? Or could other factors such as hormones play an important role in this?

We think that the sex difference in the hyperaemic response is in part attributable to physiological differences in vessel diameter. However, we could not exclude the effect of specific sex hormones on this association and, therefore, this issue should be addressed in future clinical studies specifically designed to address this question.

Question Nr 12: How could it be explained that there is no effect of antihypertensive or lipid lowering therapy? Was the lipid lowering therapy mainly consisting of statins?

Although we did not observe a significant (P≥0.35) relation between lipid-lowering therapy (all statins) and digital vasodilatation, we noticed that in our study the direction of this relationship (inverse) was similar as previously reported by the Framingham investigators (ref 6). This finding is likely attributable to indication bias in our observational, cross-sectional study. We speculate that individuals with more severe hyperlipidemia and higher risk factors burden (high blood pressure) were more often placed on drug treatment. Future randomized clinical studies are required to explore an effect of lipid-lowering or

antihypertensive medication on peripheral vascular (dys-)function.

Question Nr 13: How could it be explained that no change with age is observed?

As we explained in Discussion, similar to other studies, in which finger applanation tonometry was used to assess the endothelial function, we did not observe a significant relation between hyperaemic PPG pulse amplitude changes and age. On the other hand, previous studies reported lower hyperaemic response as assessed by FMD with advancing age. Differences in the age-related hyperaemic responses between microcirculatory and macrocirculatory reactivity might explain these divergent findings. The understanding of mechanisms which tend to preserve distal hyperaemic responses with aging required further investigations.

Question Nr 14: Given the fact that there is no correlation between brachial and digital measures of vascular function, can one conclude that the two methods do not/cannot examine the same?

As we explained in Discussion, it was already demonstrated in the previous studies that FMD and PAT provide distinct information regarding vascular function in conduit versus smaller digital vessels.

Question Nr 15: How sure can it be stated that the PPG is a useful in detection endothelial dysfunction? Should comparison with FMD ("gold standard") have been performed?

In our study, we demonstrated that measurement of the hyperaemic response by the PPG technique might be a useful tool in the detection of peripheral microvascular dysfunction associated with established risk factors such as smoking, obesity, male sex and total cholesterol. With regards to the comparison this technique with FMD, please, refer to our replies to your above comment 2. Moreover, FMD as measured by ultrasonography might be also not completely determined by endothelial function (NO production) but also could be influenced by thickness and stiffness of the vessel wall of conduit artery. On the other hand, we agree with the Reviewer that direct comparison of the PPG PWA ratio and FMD might be useful.

Question Nr 16: Concerning the stepwise multiple regression: forwards or backwards?

We performed forward stepwise multiple regression.

Question Nr 17: Were there confounders?
Question Nr 18: Fig 3 ..;"adjusted for important covariables": which covariables?

To address the comments of Reviewer on possible confounders in our study, we would like to refer to the Statistical analysis section. Briefly, important confounders/covariables of hyperaemic response identified in our study by the stepwise regression were model were sex, BMI, smoking status and total cholesterol. In footnote to Figure 3, we specified these covariables. Moreover, in our reply to comment 5 of Reviewer 1 we addressed issue of possible confounders of PPG signal.

19."several minutes" : proposal to replace by "5 minutes"
20."compared with the method based on finger applanation tonometry": proposal to write .. "compared with results from studies using the finger applanation tonomtery based method."

We addressed the minor comments 19 and 20 as suggested by Reviewer.

VERSION 2 – REVIEW

REVIEWER	Christian Delles University of Glasgow Scotland, UK
REVIEW RETURNED	08-Feb-2014

GENERAL COMMENTS	Thank you for addressing my comments so comprehensively and for making appropriate changes to the manuscript. I have no further queries.

REVIEWER	Tine De Backer UZ Ghent Dept of Cardiovascular Diseases Dept of Clinical Pharmacology Belgium
REVIEW RETURNED	16-Feb-2014

GENERAL COMMENTS	The authors did respond adequately to most of the questions. They took into account some of the comments and they changed the manuscript accordingly. However, the authors should better not use the term “endothelial function” as there is no convincing evidence that they are in fact measuring endothelial function. In fact, they provide even some evidence that they are not and that PPG is a composite measure that cannot isolate the factors influencing endothelial function. There is also no direct comparison to any measures of endothelial function. So in this stage it is better to be careful. This might also be more discussed in the discussion. The sample for the inter-session reproducibility is very small and in their answers the authors admit this. So, it is still not ok to draw any conclusions about reproducibility based on this small sample. They also do not include extreme values where more variation is most likely to be seen. The authors mention a good correlation of baseline PWA between right and left hand. The example (figure 1C) shows a marked difference in baseline flow between the two sites. Can the authors at least speculate on this finding, or choose another example if this example was not a common finding. The authors report that the results are consistent with those of other studies, in which finger applanation tonometry was used to assess the digital function and, therefore, constitute an internal validation of the PPG techniques in assessment of digital vascular function. This could be considered as a sort of validation, however, just because the results are in agreement with other findings does not mean that this can fully be used as validation. PPG responses were not different in hypertensives versus normotensives. Could the authors explain/speculate why no difference is observed.

	Concerning the question on the inverse correlation with BMI, the authors gave an answer. However, the authors did not really answer if anyway there could be a technical issue? Concerning the answer on question "the Is the sex difference mainly due to allometry? Or could other factors such as hormones play an important role in this?" If the difference is (at least partly) due to differences in vessel diameter then it is mainly due to allometric differences. This should be mentioned in the limitations because this cannot be taken into account due to the methodology.

VERSION 2 – AUTHOR RESPONSE

Reviewer 2
Question Nr 1: However, the authors should better not use the term “endothelial function” as there is no convincing evidence that they are in fact measuring endothelial function. In fact, they provide even some evidence that they are not and that PPG is a composite measure that cannot isolate the factors influencing endothelial function. There is also no direct comparison to any measures of endothelial function. So in this stage it is better to be careful. This might also be more discussed in the discussion.

The term endothelial dysfunction is widely used to describe any form of abnormal activity of the endothelium. Most commonly, endothelial dysfunction is characterized by impaired nitric oxide (NO) bioavailability (see Topic Review published in Circulation by Vita JA, 2011;124:e906-e912). Shear stress is a key activator of endothelial NO production under physiological circumstances. Most of the techniques available at present use endothelial-dependent vasomotion as the clinical endpoint for the assessment of endothelial function. Testing involves pharmacological and/or physiological stimulation (such as hyperaemic response) of endothelial release of NO and other vasoactive compounds (see Contemporary Reviews in Cardiovascular Medicine published in Circulation by Flammer et al; 2012;126:753-767).

Clinical experimental studies established a central role for endothelial NO in the augmentation of pulse wave amplitude during reactive hyperaemia measured by applanation tonometry (Nohria A et al, Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. J Appl Physiol 2006;101:545–548) or by photoplethysmography, method that we used in our study (Theunissen S, et al. Nitric oxide-related endothelial changes in breath-hold and scuba divers. Undersea Hyperb Med 2013;40:135-144). Thus, taking into account the results of previous experimental, invasive and clinical studies, and the existing expert opinion (see Review by Flammer et al; Circulation 2012), we stated in Introduction that vasodilation of the peripheral arteries during reactive hyperaemia depends in part on release of nitric oxide from endothelial cells and, therefore, reflects endothelial function. However, we agree with Reviewer that other factors (non-endothelial origin) might also contribute to the hyperaemic response. As requested by Reviewer, in the revised manuscript we substituted the term "endothelial function" by "digital vascular function" when we referred to our method.

In our study, we demonstrated that measurement of the hyperaemic response by the PPG technique might be an useful tool in the detection of peripheral microvascular dysfunction associated with established risk factors such as smoking, obesity, male sex and total cholesterol. We agree with the Reviewer that direct comparison of the PPG PWA ratio and applanation tonometry PWA or FMD might be useful. This objective could be explored in a future study specially designed to address this issue. Moreover, all other non-invasive methods for estimation of endothelial function have their limitation as well (as we highlighted in Discussion) and none of those available methods measure "exclusively" endothelial function. In our opinion, what is really important is to demonstrate in prospective studies that the hyperaemic response as assessed by the PPG technique (or by other

method) might improve identification of individuals at risk for developing cardiovascular diseases and select those individuals for more intensive preventive strategies.

Question Nr 2: The sample for the inter-session reproducibility is very small and in their answers the authors admit this. So, it is still not ok to draw any conclusions about reproducibility based on this small sample. They also do not include extreme values where more variation is most likely to be seen.

As we previously clarified in our reply to Reviewer 1, we reported the reproducibility of the average PPG PWA ratio per each 30-second time interval in each individuals. Thus, we calculated differences between 30 pairwise readings. In our reproducibility study, the mean of two measurements varied from 0.98 to 2.7. The same range we observed in the whole study population (from 0.90 to 2.8). Therefore, our reproducibility data represented the real PPG PWA variation that we observed in the general population.

Question Nr 3: The authors mention a good correlation of baseline PWA between right and left hand. The example (figure 1C) shows a marked difference in baseline flow between the two sites. Can the authors at least speculate on this finding, or choose another example if this example was not a common finding.

As suggested by Reviewer we used another example in panel C of Figure 1.

Question Nr 4: The authors report that the results are consistent with those of other studies, in which finger applanation tonometry was used to assess the digital function and, therefore, constitute an internal validation of the PPG techniques in assessment of digital vascular function. This could be considered as a sort of validation, however, just because the results are in agreement with other findings does not mean that this can fully be used as validation.

We agree with Reviewer that agreements between our findings and those reported in other studies could not be consider as a validation of the technique (it was not an objective of our study). However, we believe that these findings were not by chance and reflected an importance of measuring of the hyperaemic response by the PPG technique which is an useful tool in the detection of peripheral microvascular dysfunction associated with established cardiovascular risk factors such as smoking, obesity, male sex and total cholesterol.

Question Nr 5: PPG responses were not different in hypertensives versus normotensives. Could the authors explain/speculate why no difference is observed.

In our study we also did not observe the difference in hyperaemic response between patients with hypertension and normotensive participants. Similar finding was observed in other epidemological study (ref 10) that used applanation tonometry in assessing microvascular function. We could speculate that the PPG reactive hyperaemia index (microvasculature) is more sensitive to metabolic factors, especially body mass index, smoking and total cholesterol and less sensitive to systemic hemodynamic factors such as high blood pressure. We clarified this in Discussion (page 13).

Question Nr 6: Concerning the question on the inverse correlation with BMI, the authors gave an answer. However, the authors did not really answer if anyway there could be a technical issue?

We agree with Reviewer that a technical issue could not be completely ruled out in any study. To reduce an effect of methodological issues related to the PPG technique, we used relative changes in the PPG PWA signal in response to reactive hyperaemia rather than absolute values of PPG amplitude. Indeed, the absolute PPG PWA values could be affected by many cofactors related to the method, for instance to the differences in light transmission in subjects with and without obesity. As

we explained in Discussion, our finding on the association between the hyperaemic response and body mass index were similar to that observed in other studies, in which the different technique was used and, therefore, different technical issues could affect the results. Despite of this, the associations of reactive hyperaemia with CV risk factors were similar.

Question Nr 7: Concerning the answer on question "the Is the sex difference mainly due to allometry? Or could other factors such as hormones play an important role in this?" If the difference is (at least partly) due to differences in vessel diameter then it is mainly due to allometric differences. This should be mentioned in the limitations because this cannot be taken into account due to the methodology.

As suggested by Reviewer we added the following sentence in limitations of our study (page 14): Moreover, the sex difference in the hyperaemic response we observed in our study might be in part attributable to physiological differences in vessel diameter (allometric differences) and, therefore, could not entirely explained by low NO realise in men. Further studies should account for the differential response to hyperaemia between men and women.