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Dr Giuseppe de Luca
The Editor
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Dear Dr de Luca

We would like to thank you and the reviewers for your review of our manuscript entitled “Is Reversal of Endothelial Dysfunction Still An Attractive Target in Modern Cardiology?” (Manuscript number 7861) and your positive assessment of our manuscript pending satisfactory revision.

We have addressed the reviewers’ comments in our manuscript and the changes are appended to this letter. We have submitted both a clean copy and one with the changes highlighted in red.

We thank the editors for their consideration of our manuscript for publication and look forward to your decision.

Yours sincerely

Ify Mordi

**Response to Reviewer 1:**

The present manuscript reviews the potential role of endothelial function assessment in the routine clinical practice. Authors conclude that data are scant and its use to assess reversibility with therapeutic interventions remain en elusive clinical target. The review reports the main evidence collected so far, it is interesting but does not add a novel vision on the use of this parameter.

**1. Authors forget to state that FMD never exited the research laboratories. There are several reasons to be listed, the main one being the low reproducibility of the method when not performed according to a standardized approach and an operator-independent system of measure. Please address.**

We agree with the reviewer regarding this point and are grateful for their suggestion. We have incorporated this in our manuscript as below:

*"So why has endothelial dysfunction assessment not been adopted more widely clinically? As we have discussed, FMD appears to be the most robust and widely used technique, yet it very rarely appears in any clinical guidelines. One reason may be that although FMD does have predictive value, there are of course several other risk factors that may be easier to assess which are also predictors of adverse cardiac outcome.[31] Secondly, although many studies have reported the excellent reproducibility and variability of FMD measurement in multiple institutions[36-39], these studies all rely on following an "ideal" protocol for obtaining FMD measurements. According to a recent paper published by the European Society of Cardiology, this includes 10 minutes rest for the patient prior to measurement, correct cuff placement, an occlusion time of 5 minutes and measurement 45-60 seconds after cuff release.[40] Clearly, following this prescribed methodology takes some time and is prohibitive to its use within the clinical setting, however, not using these techniques can lead to inaccurate measurements, thus diluting the utility of FMD measurements. Automated analysis software may well overcome some of the difficulties regarding standardization of results[37], however, when it is much simpler to check a cholesterol level or measure a blood pressure, it is easy to see why FMD has perhaps not yet penetrated the clinical realm." (page 6, paragraph 2, lines 1-18)*

**2. It definitively remains an excellent research tool but it is hard to believe that it will become a routine method to assess endothelial dysfunction.**

There is no doubt that this is the case, and we feel this reinforces the timely nature of our review. To emphasize our agreement with the reviewer, we have added further reference to this in the manuscript as follows:

*Certainly, the failure of the technique to obtain widespread use in a clinical setting despite many years of use in clinical trials and a reasonable amount of prognostic evidence behind it would suggest that it may never be adopted in the cardiology community. However, the failing of FMD seems to be more due to technical issues (such as the time taken to measure it and operator variability) rather than a disbelief in its results or the importance of endothelial function. The development of PAT and interest in other aspects of endothelial function such as circulating biomarkers relating to thrombosis and inflammation may prove to be easier methods of assessing endothelial function. If an easier method could be found then (presuming it showed similar prognostic value as FMD in large-scale studies) perhaps this would have more widespread clinical applicability. (page 12, paragraph 3, lines 5-15)*

**3. The most interesting part of the review is the final one where authors address the main limitation to this method (but it could be extended to many other clinical**

**parameters) e.g. the proof that a normalization of FMD carries a prognostic benefit. This part should be extended because this is the real issue at task. Please discuss.**

We agree with the reviewer that this is the main issue regarding FMD and endothelial function assessment (and indeed part of the reason it has not yet become more widely adopted). Unfortunately, there are very few studies with a primary outcome of evaluating the prognostic benefit of endothelial dysfunction reversal. Despite a detailed literature search, we could not find any more data to add to this section. We have expanded our discussion on this section as follows:

***Does Reversal of Endothelial Dysfunction Have Any Prognostic Impact?***
*Given that several classes of drugs do seem to lead to an improvement in endothelial function, the next step is to consider whether these effects are translated into a prognostic benefit. There are however only a few studies which address this issue. Modena et al evaluated 400 post-menopausal women with hypertension and endothelial dysfunction in an attempt to assess whether an improvement in FMD using antihypertensive drugs would predict a better prognosis.[124] The authors found that improvement in endothelial function after 6 months of therapy was associated with a much reduced event rate (6% vs. 21.3% in those patients with persistently impaired endothelial dysfunction). One problem might perhaps be the fact that therapeutic options which improve endothelial function also have other beneficial effects on the cardiovascular system independent of their vasodilatory contribution. A recent study in patients with heart failure showed that patients in whom endothelial function improved following institution of optimal medical therapy had a much better prognosis than those in whom there was no improvement (hazard ratio 3.0 for those with persistently impaired endothelial function).[78]*

*Furthermore, confounding effects of medications also need to be considered – for example, hormone replacement therapy with estrogens in post-menopausal women does cause vasodilatation, however this beneficial effect is negated by their pro-thrombotic tendency. Another potential role for identification of endothelial dysfunction is that of screening. Given that there is abundant evidence to suggest that endothelial dysfunction is present before the development of clinically significant cardiovascular disease it might be beneficial to identify patients at potential risk of future events and offer disease modifying therapy. Again however this question has not yet been answered.*

*While numerous drugs that improve endothelial dysfunction have been shown to improve mortality, very few studies have specifically looked at the beneficial prognostic effects of endothelial dysfunction. This is presumably because when designing studies investigating these drugs it is very difficult to isolate the effect of endothelial dysfunction reversal given the multi-site action of drugs such as ACE inhibitors and statins. Of course, as the beneficial effects of these drugs are now well established, trials specifically looking at the prognostic benefit of endothelial dysfunction are perhaps less of a priority. (page 11, paragraphs 3-4 and page 12, paragraphs 1-2)*

**4. The manuscript would improve its readability with more tables and a real meta-analytic approach summarizing the major results available.**

We thank the reviewer for this suggestion and agree that this would enhance the manuscript. We have now added 2 tables to the manuscript detailed below (pages 29-34):

Table 1. Large studies evaluating the prognostic value of FMD.

| Author | Number of patients | Cohort | Asymptomatic Patients? | Length of follow-up (months) | Outcome | Result | Independent value of FMD? |
|---|---|---|---|---|---|---|---|
| Rossi[45] | 2,264 | Post-menopausal women | ✓ | 45 ±13 | CV death, MI, revascularisation, TIA, stroke | FMD was a predictor of MACE independently of traditional cardiac risk factors. | ✓ |
| Patti[56] | 136 | Patients with single-vessel coronary artery disease undergoing PCI | ✗ | 6 | In-stent restenosis | Patients with impaired FMD were more likely to suffer in-stent restenosis. | ✓ |
| Gokce[59] | 187 | Patients undergoing vascular surgery | ✗ | 1 | CV death, MI, unstable angina, ventricular fibrillation, stroke, raised troponin | FMD was an independent predictor of MACE in the immediate post-operative period. | ✓ |
| Brevetti[58] | 139 | Patients with peripheral arterial disease | ✗ | 23 ±10 | CV death, MI, revascularisation, TIA, critical limb ischaemia | FMD was an independent predictor of events over the follow-up period. | ✓ |
| Chan[53] | 152 | Patients with coronary artery disease | ✗ | 34 ±10 | CV death, MI, revascularisation, claudication | FMD was a strong independent predictor of risk even accounting for carotid plaque burden. | ✓ |
| Shimbo[47] | 842 | Asymptomatic multi-ethnic cohort | ✓ | 36 | Vascular death, MI, stroke | FMD was able to predict adverse events but not independently. | ✗ |
| Suzuki[43] | 819 | Asymptomatic multi-ethnic cohort including patients with metabolic syndrome | ✓ | 81 ±21 | Vascular death, MI, stroke | Patients with the combination of metabolic syndrome and endothelial dysfunction had a significantly worse | ✗ |

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| | | | | | | outcome. | |
| Yeboah[44] | 2792 | Mixed cohort of patients > 65 years | ✗ | 60 | CVD death, MI, stroke, congestive heart failure, claudication, revascularisation | FMD was an independent predictor of risk but added little to traditional risk stratification. | ✓ |
| Muiesan[57] | 172 | Hypertensive patients | ✗ | 95 ±37 | CV death, MI, revascularisation, arrhythmia, TIA, critical limb ischaemia, retinal artery occlusion | FMD below median was independently associated with adverse outcome. | ✓ |
| Shechter[46] | 618 | Healthy subjects (mixed) | ✓ | 55.2 ±21.6 | CVD death, MI, stroke, congestive revascularisation | FMD predicted adverse outcome independently. | ✓ |
| Katz[77] | 259 | Heart failure patients (LVEF <40% and NYHA class 2-3) | ✗ | 28 | Death or cardiac transplantation | FMD is associated with increased adverse outcome in ischaemic and non-ischaemic heart failure. | ✓ |

PCI – percutaneous coronary intervention; MACE – adverse major cardiovascular events; MI – myocardial infarction; TIA – transient ischaemic attack

Table 2. Selected studies examining pharmacological reversal of endothelial dysfunction

| **Author** | **Drug** | **Cohort** | **Design** | **Results** |
|---|---|---|---|---|
| Mancini[94] | Quinapril | 105 normotensive patients with coronary artery disease | Randomised double-blind, placebo controlled | Quinapril improved endothelial function compared to placebo as measured by coronary artery diameter response to acetylcholine. |
| Higashi[96] | Various ACE inhibitors, beta-blockers, calcium channel blockers and diuretics | 296 hypertensive patients | Multi-centre cohort study | ACE inhibitors significantly improved endothelial dependent vasodilatation compared to other drug classes as measured by forearm blood flow. |
| Wassmann[97] | Candesartan, felodipine | 47 patients with high cholesterol | Randomised double-blind, placebo controlled | Candesartan improved forearm blood flow compared to felodipine or placebo |
| Ghiadoni[98] | Nifedipine, amlodipine Perindopril, | 168 patients with hypertension | Randomized, single-blind, parallel-group | Only perindopril improved FMD (although perindopril, telmisartan, |

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|---|---|---|---|---|
| | telmisartan, atenolol, nebivolol | | | nifedipine and amlodipine reduced oxidative stress and increased plasma antioxidant capacity). |
| Tzemos[99] | Valsartan, amlodipine | 25 hypertensive patients | Randomised double-blind, crossover | Valsartan improved forearm blood flow. |
| Takagi[100] | Telmisartan | Mixed; 398 patients | Meta-analysis of 7 studies | Statistically significant increase in FMD by 48.7%. |
| Farquaharson[101] | Spironolactone | 10 patients with NYHA class I-II heart failure | Randomised, double-blind placebo-controlled crossover study | Spironolactone improved forearm blood flow compared to placebo. |
| MacDonald[103] | Spironolactone | 43 patients with NYHA class I-II heart failure | Randomised, double-blind crossover study | Spironolactone improved forearm blood flow compared to placebo. |
| Abiose[104] | Spironolactone | 20 patients with NYHA class III-IV congestive heart failure | Cohort study | Spironolactone improved FMD at 4 weeks with a sustained improvement at 8 weeks. |
| Tzemos[107] | Nebivolol, atenolol | 12 hypertensive patients | Randomised, double-blind crossover study | Only nebivolol was able to improve endothelial dependent vasodilation. |
| Pasini[108] | Nebivolol, atenolol | 40 hypertensive patients with 40 controls | Randomised double-blind parallel group | FMD improved only in the group treated with nebivolol. |
| Matsuda[109] | Carvedilol | 29 patients with coronary artery disease | Randomised, placebo controlled | Carvedilol significantly improved FMD after 4 months treatment. |
| Agewall[116] | Atorvastatin | 20 healthy smokers, 20 healthy non-smokers | Open label placebo controlled randomised cross-over | Smokers had a lower baseline FMD. Atorvastatin improved FMD in smokers but had no effect in non-smokers. |
| Ostad[117] | Atorvastatin, ezetimibe | 58 patients with coronary artery disease | Double-blind, randomised, parallel group | High-dose atorvastatin improved FMD significantly more than low dose atorvastatin + ezetimibe independently of improvement in LDL cholesterol. |
| Gounari[118] | Rosuvastatin, ezetimibe | Patients with heart failure | Double-blind, placebo controlled, cross-over trial | Rosuvastatin caused a significant improvement of FMD compared to ezetimibe and independent of LDL cholesterol and baseline brachial artery diameter. |
| Pitocco[121] | Metformin | 42 type 1 diabetics without | Randomised double-blind, | Significant improvement in FMD by 1.32% |

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| | | overt cardiovascular disease | placebo controlled | compared to placebo. |
| Lamendola[122] | Ranolazine | 30 type 2 (non-insulin dependent) diabetics without overt cardiovascular disease | Randomised double-blind, placebo controlled | Significant improvement in FMD compared to placebo after 2 weeks of ranolazine therapy. |
| Kao[123] | Allopurinol | 67 patients with CKD stage 3 and LV hypertrophy | Randomized, double-blind, parallel-group | Significant improvement in FMD compared to placebo after 9 months of allopurinol therapy. |

**5. Data and survival curves in the prognosis sub-session should be reported.**

We thank the reviewer for this suggestion. We have included some data as follows:

*"The authors found that improvement in endothelial function after 6 months of therapy was associated with a much reduced event rate (6% vs. 21.3% in those patients with persistently impaired endothelial dysfunction)." (page 11, paragraph 3, lines 6-9)*

*"A recent study in patients with heart failure showed that patients in whom endothelial function improved following institution of optimal medical therapy had a much better prognosis than those in whom there was no improvement (hazard ratio 3.0 for those with persistently impaired endothelial function).[78]" (page 11, paragraph 3, lines 11-15)*

Unfortunately we have not received permission to use the curves from other studies.

**6. It would be interesting to know how these authors use FMD in patient assessment.**

We do not routinely used FMD in our unit for clinical purposes, and we have answered this question as below.

*"Indeed, in our unit, FMD is only used in research studies and is not used at all clinically." (page 12, paragraph 3, lines 15-16)*

**Response to Reviewer 2:**

**Is Reversal of Endothelial Dysfunction Still An Attractive Target in Modern Cardiology?**

Mordi and Tzemos provide a brief and somewhat simplistic view of endothelial function and dysfunction and its measurement and apparent clinical role.

There are a number of issues with this manuscript that should be addressed.

**1. (minor, p. 3, line 3 and following text). The authors state that Furchgott and Zawadski (1980) made their observation in 'large arteries of rabbits'; with the actual observation being made after Jellife (1962, JPET, 135:349-353). However, on p. 376, 3rd paragraph of the 1980 paper, a role for their observation is also made with reference to large and small vessels from cat, dog, guinea pig and rat. This issue is commonly overlooked but please correct this text.**

We thank the reviewer for noticing this error and apologise. We have amended this as follows:

*"However, discovery by Furchgott and Zadawski that the large blood vessels of mammals only dilate if the endothelium is intact due to its response to nitric oxide (NO) was the first step in our understanding that the endothelium is a key modulator of cardiovascular health.*[1]*" (page 3, paragraph 1, lines 2-6)*

**2. (major, p. 3, latter half of 1st paragraph). The authors state that vasodilation in healthy human endothelium is due to nitric oxide (NO). Whilst this is in part correct, the text implies that this is the only mechanism involved and this potentially naive and misleading; and is obviously not correct. There are well described defects in other endothelium-dependent relaxation (EDR) pathways in studies of human artery function in health and disease; as well as defects in ED constricting factor (CF) mechanisms? The latter are alluded to in the 1st sentence of the 2nd paragraph, and elsewhere in the text (eg. p. 7, 1st and 3rd paragraphs; p. 8, 4th paragraph; XXX), but this contradicts the first paragraph at p. 3. Please expand the text at p. 3 to accurately reflect the literature. I believe that a simple fix is to add a paragraph mentioning the other mechanisms and then to state that this review will focus on NO.**

We thank the reviewer again for this correction. We have amended the manuscript as follows:

*"The healthy human endothelium maintains a vasodilated state as a baseline, in part due to NO production from L-arginine by nitric oxide synthase." (page 3 paragraph 1 lines 9-11)*

*"Indeed, although NO is the main endothelium-derived relaxing factor there are other factors active on the endothelium, all of which play a key role in its health. Other endothelium-derived relaxing factors include prostacyclin and endothelium-derived hyperpolarizing factor, both of which can show increased activity in response to a decrease in NO. Meanwhile, there are several endothelium-derived contracting factors causing vasoconstriction such as endothelin-1, thromboxane $A_2$ and prostaglandin $H_2$. Nevertheless, the majority of clinical studies have concentrated on NO, and this will be the focus of our review." (page 3 paragraph 1 lines 17-24)*

**3. (minor, p. 4, line 8). Please insert 'apparently' before 'paradoxical'; and insert 'smooth muscle' after 'direct'; and at the end of this sentence add ', at comparatively high concentrations'. That is, the constrictor effects of Ach are due to smooth muscle**

**M1 activation and occur at >~[10-4 M], whilst the endothelial effects occur at M3 at <~[10-4 M]. The current text is not informative on this point.**

We agree with the reviewer's changes and have amended the manuscript as suggested:

*"In dysfunctional coronary arteries Ach causes reduced vasodilatation or apparently paradoxical vasoconstriction due to the unopposed direct smooth muscle muscarinic action of Ach at apparently high concentrations.[9]" (page 4, paragraph 2, lines 9-12)*

**4. (major, p. 4, line 16 and related to point 2, above). Please correct the implication that endothelial function is analogous to NO bioactivity, as, although it can be in some vessels, it is not in all beds. Again, as per point 2, above, please avoid use of generalizations that are incorrect.**

The reviewer is correct to point this out and we agree. We have corrected this as follows:

*"Endothelial function, which is closely related to NO biocactivity, can be measured by constructing dose-response curves to escalating doses of Ach and measuring the rate of change in arm swelling by strain gauge." (page 4, paragraph 3, lines 7-10)*

**5. (minor, p. 6, line 15). Please cite references referred to after 'FMD'.**

The reviewer's request is not entirely clear. We are happy to adjust as required however.

**6. (minor). A Figure illustrating the aim/s / summary / conclusion of the review would be helpful.**

We thank the reviewer for this suggestion and have added this as follows (page 2--3). This will also serve as our core tip:

*Figure 1. Summary of Review*

**Aims**

- To explore the evidence for reversal of endothelial dysfunction and assess its clinical relevance.

**Summary**

- Endothelial dysfunction is widely prevalent and has been to have prognostic significance in prediction of adverse cardiovascular events in various cardiovascular conditions and potentially in asymptomatic patients.
- Various drugs have shown the ability to reverse endothelial dysfunction, with the most evidence being for drugs acting on the renin-angiotensin-aldosterone system.
- Despite this, very few studies have set out primarily to identify whether reversal of endothelial dysfunction actually has any prognostic benefit.

**Conclusions**

- While there is ample evidence to suggest that endothelial dysfunction has prognostic significance and that it can be reversed, we still do not know whether it is a clinically relevant independent therapeutic target or simply a marker of adverse cardiovascular outcome.
- Limitations of the techniques for assessing endothelial function have also meant that it has not yet been widely adopted in the clinical arena.