

## BRIEF ULTRARAPID COMMUNICATION

### Kidney Transplantation in a Patient Lacking Cytosolic Phospholipase A<sub>2</sub> Proves Renal Origins of Urinary PGI-M and TX-M

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**Running title:** Renal Origin of PGI-M and TX-M



# Circulation Research

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## ABSTRACT

***Rationale:*** The balance between vascular prostacyclin which is anti-thrombotic and platelet thromboxane A<sub>2</sub> which is pro-thrombotic is fundamental to cardiovascular health. Prostacyclin and thromboxane A<sub>2</sub> are formed following the concerted actions of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>α) and cyclooxygenase. Urinary 2,3-dinor-6-keto PGF<sub>1α</sub> (PGI-M) and 11-dehydro-TXB<sub>2</sub> (TX-M) have been taken as biomarkers of prostacyclin and thromboxane A<sub>2</sub> formation with the circulation and used to explain cyclooxygenase biology and patient phenotypes, despite concerns that urinary PGI-M and TX-M originate in the kidney.

***Objective:*** We report data from a remarkable patient carrying an extremely rare genetic mutation in cPLA<sub>2</sub>α, causing almost complete loss of prostacyclin and thromboxane A<sub>2</sub>, who was transplanted with a normal kidney resulting in an experimental scenario of ‘*whole body cPLA<sub>2</sub>α knockout, kidney specific knock-in*’. By studying this patient, we can determine definitively the contribution of the kidney to the productions of PGI-M and TX-M and test their validity as markers of prostacyclin and thromboxane A<sub>2</sub> in the circulation.

***Methods and Results:*** Metabolites were measured using LC-MS/MS. Endothelial cells were grown from blood progenitors. Before kidney transplantation the patient’s endothelial cells and platelets released negligible levels of prostacyclin (measured as 6-ketoPGF<sub>1α</sub>) and thromboxane A<sub>2</sub> (measured as TXB<sub>2</sub>), respectively. Likewise, the urinary levels of PGI-M and TX-M were very low. Following transplantation and the establishment of normal renal function the levels of PGI-M and TX-M in the patient’s urine rose to within normal ranges while endothelial production of prostacyclin and platelet production of thromboxane A<sub>2</sub> remained negligible.

***Conclusions:*** This data shows that PGI-M and TX-M can be derived exclusively from the kidney without contribution from prostacyclin made by endothelial cells or thromboxane A<sub>2</sub> by platelets in the general circulation. Previous work relying upon urinary metabolites of prostacyclin and thromboxane A<sub>2</sub> as markers of whole body endothelial and platelet function now requires re-evaluation.

### **Key words:**

Prostacyclin, thromboxane A<sub>2</sub>, PGI-M, COX-2, blood outgrowth endothelial cells, endothelial function,

### **Nonstandard Abbreviations and Acronyms:**

6-ketoPGF <sub>1α</sub>	6-keto-prostaglandin F <sub>1α</sub>
cPLA <sub>2</sub> α	group IV A cPLA <sub>2</sub> α
IL-1β	interleukin-1β
NSAID	nonsteroidal anti-inflammatory drug
PGI-M	2,3-dinor-6-keto PGF <sub>1α</sub>
TXB <sub>2</sub>	thromboxane A <sub>2</sub>
TX-M	11-dehydro-TXB <sub>2</sub>

## INTRODUCTION

For over 40 years the importance of balance in the production of prostanoids has been a central theme in the understanding of cardiovascular health. Attention has focused upon prostacyclin derived from the vasculature, which is anti-thrombotic and a vasorelaxant and thromboxane A<sub>2</sub> derived from platelets, which is pro-thrombotic and a vasoconstrictor. Both prostacyclin and thromboxane A<sub>2</sub> are formed following the concerted actions of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>α) and cyclooxygenase (COX). COX is present in two isoforms; COX-1 is constitutively expressed throughout the body<sup>1-4</sup> whilst COX-2 is present constitutively only in discreet regions of the body, which include the kidney<sup>5-8</sup>. COX-2 is also expressed at the site of inflammation and in cancer and as such is the therapeutic target for the nonsteroidal anti-inflammatory group of drugs (NSAIDs), which include aspirin, ibuprofen and celecoxib.

It was found early on in prostanoid research that both prostacyclin and thromboxane A<sub>2</sub> are very short-lived within the circulation and that measurements of either of them or their immediate metabolites were relatively uninformative. The establishment of analytical techniques to measure 2,3-dinor-6-keto-PGF<sub>1α</sub> (PGI-M), a stable metabolite of prostacyclin and 11-dehydro-TXB<sub>2</sub> (TX-M), a stable metabolite of thromboxane A<sub>2</sub> in urine therefore appeared to provide the possibility of useful biomarkers of cardiovascular health and of drug action. However, for PGI-M and TX-M to work as biomarkers their levels in urine should reflect levels in the circulation and whilst this idea has been suggested based on selective inhibition of urinary TX-M with aspirin<sup>9</sup> it has not been experimentally proven. Since this early work it has been generally assumed that PGI-M and TX-M measured in urine reflected levels in the cardiovascular system as a whole, dependent upon prostacyclin production by endothelial cells and thromboxane A<sub>2</sub> production by platelets<sup>10-15</sup>. This assumption has now become dogma and stable urinary metabolites of prostacyclin and thromboxane A<sub>2</sub> have been used in many studies; e.g. as of September 2017 an on-line search on *PubMed* with the terms 'urinary prostacyclin metabolite' or 'urinary thromboxane metabolite' returns over 300 and 400 papers respectively, while search of *clinicaltrials.gov* with the term 'urinary prostanoid' produces 48 entries. Results from these studies have apparently informed (i) drug action in clinical studies<sup>11</sup>, (ii) personal risk of cardiovascular disease in patient groups<sup>14</sup> and (iii) a plethora of basic science relating to eicosanoids. A widely-held concept derived from such studies is that prostacyclin released by endothelial cells is formed through the actions of COX-2, following from the observation that COX-2-selective drugs reduce PGI-M and relying upon the assumption that PGI-M reflects the production of prostacyclin by endothelial cells<sup>12, 15, 16</sup>. However, this idea is not universally accepted<sup>1, 2, 5, 6, 8, 16</sup> since conflicting observations indicate that COX-1<sup>1-4, 8, 16, 17</sup> is the dominant isoform within the vasculature including endothelial cells leading some of us to suggest that urinary markers of prostacyclin can be derived from the kidney<sup>1</sup> where COX-2 is highly expressed<sup>5-7</sup>.

To date there have been no definitive models in which the renal origin of PGI-M and TX-M can be tested. However, here we present a report of a patient with inherited human group IV A cPLA<sub>2</sub>α deficiency<sup>18</sup>, previously found by our group to almost completely lack the vital capacity to form a number of eicosanoids including endothelial prostacyclin and platelet thromboxane A<sub>2</sub><sup>18, 19</sup>. In 2015 the patient underwent a kidney transplant receiving a normal cPLA<sub>2</sub> sufficient organ. The transplant has resulted in the serendipitous generation of a remarkable experimental model akin to a human '*whole body cPLA<sub>2</sub>α knockout, kidney specific knock-in*'. Now, for this patient, we can determine definitively the contribution of the kidney to the production of PGI-M and TX-M and so test the relevance of these measurements as markers of prostacyclin and thromboxane A<sub>2</sub> in the circulation.

## METHODS

The authors declare that all supporting data are available within the article.

### *Patient details.*

The patient (female, of Serbian heritage, born 1966) presented at the age of 2 years with peptic ulceration, bleeding and pyloric stenosis, which required pyloroplasty and selective vagotomy. The patient went on to have a life long history of gastrointestinal disease<sup>18</sup> culminating in the diagnosis of CMUSE<sup>18</sup>. In 2014 we reported that the patient carries a homozygous 4 bp deletion (g.155574\_77delGTAA) in the *PLA2G4A* gene resulting in a frameshift of 10 amino acids before a premature stop codon (p.V707fsX10) and the loss of 43 amino acids (residues 707–749) at the C-terminus of group IV A cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>α). This mutation results in a complete loss of cPLA<sub>2</sub>α protein expression. In line with loss of cPLA<sub>2</sub>α, generation of eicosanoids by whole blood<sup>18</sup>, isolated platelets, peripheral blood monocytes or blood outgrowth endothelial cells obtained from the patient<sup>19</sup> were dramatically reduced. Plasma and urinary levels of most eicosanoids were also accordingly much lower than the normal range in samples from the patient<sup>18,19</sup>. In 2014 renal function of the patient declined due to tubulointerstitial nephritis leading to end stage renal failure requiring dialysis during which time the patient was producing ≈1l/day of urine. In 2015 the patient underwent a renal transplant receiving a live unrelated spousal donor kidney. After the kidney transplant had stabilized, blood and urine samples were collected for analysis using liquid chromatography-tandem mass spectrometry at 1-3 months post-transplant. Blood outgrowth endothelial cells were also isolated after transplant and samples collected for eicosanoid measurements after stimulation in culture. The patient received tacrolimus as anti-rejection therapy.

### *Blood collection and ethics.*

Blood was collected by venepuncture and urine samples as midstream flow from healthy volunteers and the patient bearing a homozygous mutation in the *PLA2G4A* gene, which disrupts the active site of cPLA<sub>2</sub>α<sup>18</sup>.

### *Whole blood stimulation.*

Heparin anti-coagulated whole blood was incubated with vehicle (PBS) or Horm collagen (Nycomed, St Peter, Austria). TXB<sub>2</sub> levels were measured by gas chromatography-tandem mass spectrometry (LC-MS/MS) in the conditioned plasma.

### *Endothelial cells.*

Blood outgrowth endothelial cells were grown out from progenitors in human blood as previously described<sup>20-23</sup>. Once colonies emerged (between days 4 and 20) cells were expanded and maintained in Lonza EGM-2 media (Lonza, Slough, UK) + 10% FBS and experiments performed between passages 2 and 8.

Cells were plated on 48- or 96-well plates. For eicosanoid measurements endothelial cells were primed with IL-1β (1 ng/mL; Invitrogen, Life Technologies, Paisley, UK) to up-regulate COX pathways as described previously<sup>24</sup> before being treated for 30 minutes with the calcium ionophore A23187 to activate PLA<sub>2</sub>.

### *Eicosanoid analysis.*

Levels of prostanoids in urine, whole blood and endothelial cell samples were determined by LC-MS/MS as previously described<sup>1, 19, 25, 26</sup>.

### *Statistics and data analysis.*

Data are shown as individual data points.

### **Study approval.**

All experiments were subject to written informed consent, local ethical approval (healthy volunteer samples for platelet/leucocyte studies; St Thomas's Hospital Research Ethics Committee, reference 07/Q0702/24: endothelial cell studies; Royal Brompton & Harfield Hospital Research Ethics Committee, reference 08/H0708/69: patient samples; South East NHS Research Ethics Committee) and in accordance with Declaration of Helsinki principles.

## **RESULTS**

Before the kidney transplant, the patient had developed end stage kidney failure with urine production of  $\approx 1$ l/day requiring haemodialysis three times a week. Post-operative recovery following transplant was uneventful. Her renal function normalized with blood urea nitrogen of 6.6 and creatinine of 88  $\mu\text{mol/litre}$  by 4 weeks post-transplant.

In healthy volunteers PGI-M and TX-M tend to be higher in females than males<sup>19</sup>. However, in line with her condition, before the transplant levels of PGI-M and TX-M in the patient's urine were low and well below levels in control donors and the published<sup>27</sup> normal ranges (Figures 1)<sup>18, 19</sup>. Remarkably the new kidney restored levels of urinary PGI-M and TX-M to the normal range. This phenomenon was found to be selective to the kidney because the ability of endothelial cells from the patient to produce prostacyclin and of platelets from the patient to produce thromboxane A<sub>2</sub> remained low and unchanged by the kidney transplant (Figure 1). Similarly, there was no increase in the levels of PGI-M within the circulation, but rather a small reduction ( $25\pm 3\%$ ), when plasma samples from after transplantation (n=5) were compared to those from before transplantation (n=8).

## **DISCUSSION**

Here we describe a remarkable clinical and experimental situation, the serendipitous generation of a unique human model in which to explore the origins of the urinary eicosanoid metabolites of prostacyclin (PGI-M) and thromboxane (TX-M), previously claimed to be representative of global, whole body, endothelial prostacyclin and platelet thromboxane A<sub>2</sub> production.

Because eicosanoids, including prostacyclin, protect the gastrointestinal tract and the kidney, the long term clinical symptoms of the patient can be entirely explained by the genetic deficiency and the associated lack of cPLA<sub>2</sub> $\alpha$  activity, illustrating the powerful protective role that eicosanoids play in homeostasis. After receiving a genetically normal kidney the patient continued to be almost entirely unable to produce prostacyclin and thromboxane A<sub>2</sub> from her endothelial cells and platelets. However, despite the continuing absence of endothelial prostacyclin production and platelet thromboxane A<sub>2</sub> production after transplant, the patient's urine contains apparently normal<sup>27</sup> levels of PGI-M and TX-M. Importantly, it has already been demonstrated that the use of tacrolimus to reduce organ rejection in renal transplant patients is not associated with changes in either PGI-M or TX-M<sup>28</sup>. It is therefore impossible in this patient that PGI-M and TX-M were derived from, or reflective of, endothelial and platelet eicosanoid productions.

These results not only describe a unique clinical case of organ transplantation in a patient with an incredibly rare gene deletion, but also show unequivocally that the kidney alone can support the production of normal levels of PGI-M and TX-M and that these cannot be assumed as markers for prostacyclin and thromboxane A<sub>2</sub> production within the cardiovascular system as a whole. While there may be concerns that the patient presented here has very particular pathologies, which may not speak for normal physiological

function, the same can be said for any of the many patients across a wide range of diseases in which measurement of PGI-M and TX-M have been used to describe clinical conditions.

Importantly as mentioned above, PGI-M has been used to define the idea that endothelial cells produce prostacyclin through the action of COX-2<sup>15</sup> since selective inhibitor drugs, such as celecoxib, reduce urinary PGI-M<sup>11</sup>. However, this idea is not universally accepted<sup>2, 8, 29, 30</sup> and has not been supported by direct evidence, which instead identifies the ubiquitously expressed constitutive form, COX-1 as the principle driver of prostacyclin in the circulation<sup>1, 2, 30</sup>. Similarly, there have been anomalies in the rationale that TX-M accurately reflects thromboxane A<sub>2</sub> in the circulation. For example early studies demonstrated that platelet thromboxane A<sub>2</sub> production could be strongly inhibited without a concomitant reduction in urinary TX-M<sup>31</sup>. At the time this was taken as indicating the need for substantial platelet COX inhibition to reduce in vivo platelet activation. Our data now provide definitive proof for the alternative, and simpler, conclusion that both urinary PGI-M and TX-M originate from the kidney and are not necessary reflective of prostacyclin and thromboxane A<sub>2</sub> in the circulation.

In conclusion, we now need to reconsider the many studies and clinical trials that have used measures of PGI-M and TX-M to construct some of the fundamental concepts of eicosanoid biology and to characterize various patient groups. This is particularly important in the areas of aspirin therapy and COX-2 biology where urinary markers have been used to inform discussions upon the mechanisms associated with NSAIDs and cardiovascular risk<sup>12, 15, 16</sup>. In the light of our findings, which prove that urinary PGI-M can originate from the kidney, we may conclude that earlier studies showing COX-2 inhibitor drugs to reduce PGI-M simply confirm the kidney as a prime site for constitutive COX-2 expression and add to the idea that blockade of the production of protective COX-2 derived prostanoids in the kidney contributes to NSAID-induced cardiovascular side effects.

#### **SOURCES OF FUNDING**

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#### **AUTHOR CONTRIBUTIONS**

RBK, NSK, DMR, MLE, WEW, MVC and GLM performed the research; JAM, RBK, NSK, DMR, MLE, GLM, DCZ and TDW analyzed the data; RBK, NSK, DMR, MLE, WEW, MVC, HL, MMY, GLM and DCZ edited the paper; JAM, MMY and TDW designed the research; JAM and TDW wrote the paper.

#### **DISCLOSURE**

Authors report no conflicts of interest.

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## FIGURE LEGENDS

**Figure 1.** A – Production of prostacyclin measured as 6-keto-PGF<sub>1α</sub> from endothelial cells stimulated with calcium ionophore (A23187); B – urinary levels of the prostacyclin metabolite PGI-M (2,3-dinor-6-keto-PGF<sub>1α</sub>); C – production of thromboxane A<sub>2</sub> measured as TXB<sub>2</sub> from whole blood stimulated with collagen; D – urinary levels of the thromboxane metabolite TX-M (11-dehydro-TXB<sub>2</sub>). Measurements made in samples from healthy volunteers and from the patient before and after kidney transplantation. Results from healthy volunteers and cPLA<sub>2α</sub>-deficient patient pre-op includes data previously published<sup>19</sup>.



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## NOVELTY AND SIGNIFICANCE

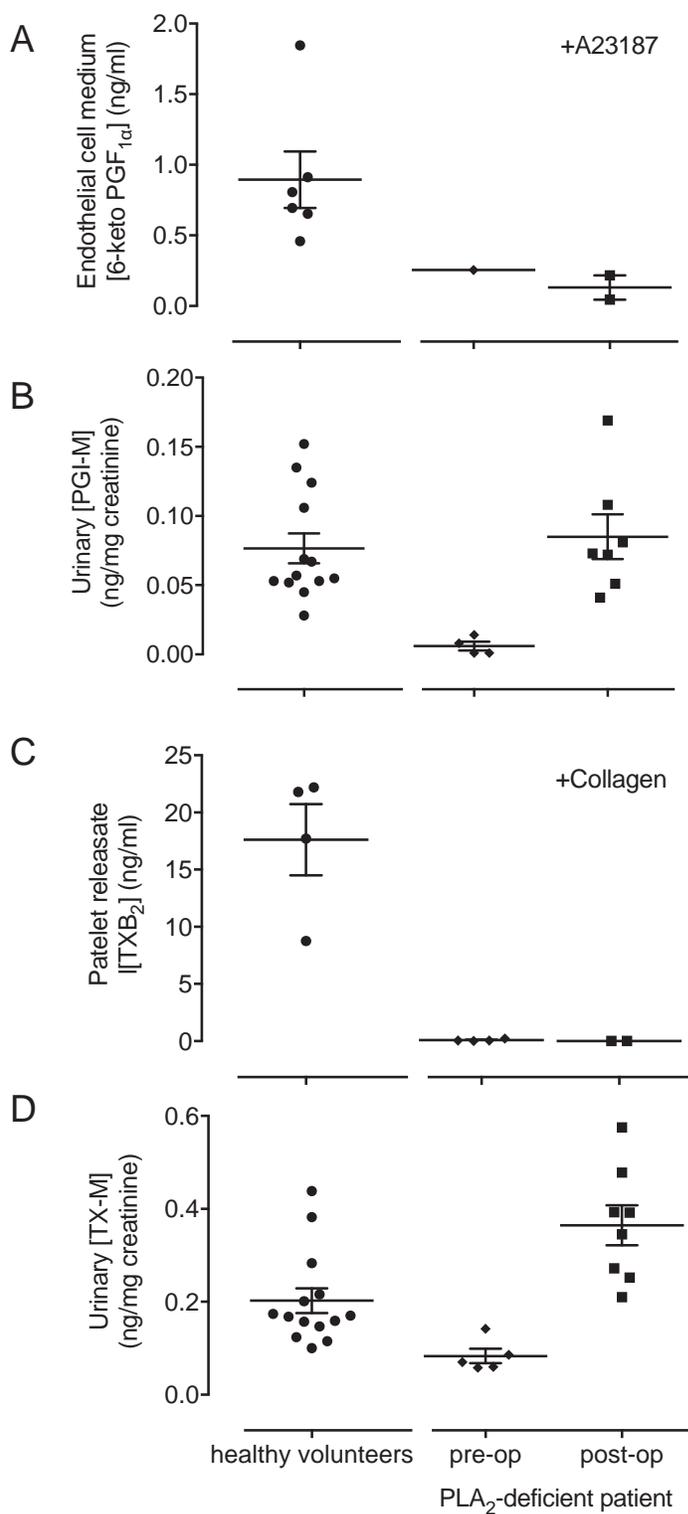
### *What Is Known?*

- The balance of formation of the cyclooxygenase derived eicosanoids, vascular prostacyclin, which is anti-thrombotic, and platelet thromboxane,  $A_2$  which is pro-thrombotic, lies at the center of cardiovascular health.
- It has been widely assumed that the production of these two short-lived mediators can be followed by the measurement of stable urinary metabolites, 2,3-dinor-6-keto  $PGF_{1\alpha}$  (PGI-M) for prostacyclin and 11-dehydro-TXB<sub>2</sub> (TX-M) for thromboxane  $A_2$ , and these measurements have been used to explain cyclooxygenase biology and patient phenotypes.

### *What New Information Does This Article Contribute?*

- Study of the urinary metabolites of a unique patient lacking the ability to form vascular prostacyclin and platelet thromboxane  $A_2$  who received a kidney transplant demonstrated unequivocally that PGI-M and TX-M can be derived exclusively from the kidney.
- These findings suggest re-evaluation of the urinary metabolites of prostacyclin and thromboxane  $A_2$  as markers of whole body endothelial and platelet function.

The balance between vascular prostacyclin which is anti-thrombotic and platelet thromboxane  $A_2$  which is pro-thrombotic is fundamental to cardiovascular health. However, both these mediators are very short lived and cannot be directly measured in circulation. Hence, researchers have relied upon 2,3-dinor-6-keto  $PGF_{1\alpha}$  (PGI-M) and 11-dehydro-TXB<sub>2</sub> (TX-M) as urinary biomarkers. These measures have been used to explain cyclooxygenase biology despite the lack of definitive proof of their sources. We studied a unique patient carrying an extremely rare genetic mutation in group IV A cytosolic phospholipase  $A_2$  (cPLA<sub>2</sub> $\alpha$ ), causing almost complete loss of prostacyclin and thromboxane  $A_2$ , who was transplanted with a normal kidney. Before kidney transplantation the patient's endothelial cells and platelets released negligible levels of prostacyclin and thromboxane  $A_2$ , respectively, accompanied with very low levels of PGI-M and TX-M. Following transplantation, the levels of PGI-M and TX-M in the patient's urine rose to within normal ranges without any increases in the production of prostacyclin and thromboxane  $A_2$ . These findings demonstrate that PGI-M and TX-M can be derived exclusively from the kidney and suggest that literature relying upon the measurement of these metabolites needs to be re-examined.



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