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Neutrophil gelatinase-associated lipocalin (NGAL) is localised to the primary cilium in renal tubular epithelial cells - A novel source of urinary biomarkers of renal injury

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ABSTRACT

Background: Primary cilia have been shown to play a central role in regulating epithelial cell differentiation during injury and repair. Growing evidence implicates structural and functional abnormalities of primary cilia in kidney epithelial cells in the onset and development of various kidney diseases including polycystic kidney disease (PKD). Neutrophil-gelatinase associated lipocalin (NGAL) has been identified as a reliable urinary biomarker of kidney injury. However, the mechanism by which this protein accumulates in patient urine samples has not been fully elucidated.

Methods: Human renal tubular epithelial cells (RPTECs) were exposed to previously characterized deciliating agents to assess mechanisms of primary cilium loss. Confocal immunofluorescent imaging was employed to visualise the effects on cilia. Western blot analysis was utilised to quantify the ciliary protein Arl13b in both RPTEC whole cell lysates and supernatants. Co-immunoprecipitation was used to demonstrate co-localisation of Arl13b and NGAL in urinary samples from a clinical Chronic Allograft Nephropathy (CAN) cohort.

Results: Immunofluorescent analysis revealed that NGAL was localised to the primary cilium in RPTECs, co-localizing with a ciliary specific protein, Arl13b. Deciliation experiments showed that loss of the cilia coincided with loss of NGAL from the cells.

Conclusion: The accumulation of NGAL in supernatants *in vitro* and in the urine of CAN patients was concurrent with loss of Arl13b, a specific ciliary protein. The findings of this study propose that increased NGAL urinary concentrations are directly linked to deciliation of the renal epithelial cells as a result of injury.

1. Introduction

The primary cilium is a sensory organelle found on the apical surface of almost every mammalian cell type, including renal tubular epithelial cells [1,2]. Structurally, this non-motile cilium consists of an axoneme of nine doublet microtubules which are assembled from the basal body, and in turn anchors the cilium at the cell surface [3]. Since their initial discovery in the 1960s [4], the primary cilium has progressed from an unknown entity to being regarded as a key organelle, central to cell structure and a variety of cellular functions including mechano- and chemo- sensation, developmental signalling pathways and a wide variety of human diseases.

Renal epithelial cell primary cilia function as sensory organelles, projecting into the tubular lumen of the nephron, monitoring fluid flow and initiating signalling pathways central to the maintenance of a normal epithelial phenotype and organ homeostasis [5–7]. In the context of the kidney, primary cilia are essential for the maintenance of normal renal tubular architecture and organ homeostasis and must be

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disassembled in order for the renal cell to proceed through the mitotic cycle. In the renal tubules, complete loss of primary cilia or dysfunctional cilia leads to the uncontrolled proliferation of tubule cells and the formation of cysts [8]. Previous studies have implicated defects in the key components of the renal primary cilia in an increasing array of different renal disease; for example polycystin-1 and polycystin-2 in human autosomal dominant polycystic kidney disease (PKD), BBS family proteins in Bardet-Biedl syndrome (BBS), nephrocystin family proteins in nephronophthisis, MSK-1 in Meckel-Gröber syndrome and Arl13b in Joubert syndrome [3,9–14].

NGAL or lipocalin 2 is a 25 kDa member of the lipocalin family of extracellular binding proteins, which interact with cell-surface receptors and play a role in ligand transport [15–18]. NGAL urinary levels in a healthy individual are typically low, < 5 ng/ml, due to resorption from the filtrate by the proximal tubular cells. During renal tubular injury, increased secretion and decreased reabsorption is apparent, as evidenced by increasing urinary NGAL concentrations. NGAL levels have been found to be significantly increased in patients with tubular pathologies [19]. Previous studies have indicated that NGAL measurement may be useful for evaluating drug induced renal injury or nephrotoxicity [20], and increased NGAL levels have been linked to the progression of Chronic Allograft Nephropathy (CAN), also known as Chronic Allograft Injury (CAI), in patients, in part driven by the known nephrotoxicity of the various immunosuppressant drugs the patients are required to take [18,21]. The aim of this study was to investigate the subcellular localisation of NGAL in human RPTEC cells, to gain insight into potential mechanisms underlying urinary NGAL elevations during renal damage.

2. Results

2.1. Differentiated renal epithelial proximal tubule cells express a single primary cilium

Human Renal Proximal Tubular cells (RPTEC/TERT1) were differentiated for 10 days post-confluency on 6 well plates. Previous work conducted in our lab demonstrated the presence of cilia on the RPTEC/TERT1 cells, with primary cilia extending above the apical surface of the RPTEC/TERT1 cells [22], with primary cilia observed in over 90% of the cells in the confluent monolayer (Fig. 1a (iii)). To further characterise the primary cilia of the RPTEC/TERT1 cell line, immunofluorescent confocal microscopy was utilised. Acetylated α-tubulin was immunofluorescently stained with Texas red, demonstrating the presence of acetylated α-tubulin structures or cilia projecting above the apical cell surface (Fig. 1a (i)). Cytoplasmic microtubules were also visualised; however, this cytoplasmic network was located on a different plane to those microtubules comprising the primary cilium. Immunofluorescent FITC staining of Zona Occludens (ZO-1), a well characterized epithelial junctional protein, was employed to identify the cellular borders (Fig. 1a (ii)). The resulting image obtained by merging the red and green channels demonstrates that each cell expressed a single primary cilium (Fig. 1a (iii, iv)).

2.2. NGAL co-localizes with acetylated Arl13b in the primary cilium

Following Immunofluorescent microscopy confirmation of the presence of primary cilia on the RPTEC/TERT1 cells *in vitro*, further investigations were conducted to investigate the localisation of NGAL within the renal epithelial cells. Immunofluorescent FITC staining of Arl13b, a well characterized cilia-specific protein, identified the cilia structure (Fig. 1b (i, iv)), while Texas red staining was employed to identify NGAL expression in the RPTEC/TERT1 cells (Fig. 1b (ii, v)). This alternative ciliary protein was chosen as it is known that ARL13b expression, unlike acetylated alpha tubulin, is restricted to the primary cilium, making it a highly specific cilia marker. As a result of the cilia specificity of Arl13b no cytoplasmic microtubules were identified, in contrast to the previous confocal microscopy investigation utilising acetylated alpha tubulin (Fig. 1b (i)). Merging the two immunofluorescent channels demonstrated that NGAL co-localised with Arl13b, thus suggesting that NGAL is expressed in the primary cilium (Fig. 1b (iii–vi)).

2.3. Primary cilia can be isolated from RPTEC/TERT1 cells

RPTEC/TERT1 cells were differentiated for 10 days post-confluency in 150 mm culture dishes. Poly-L-Lysine slides were used to strip the cilia from the cell surface as previously described [23] (Fig. 2a (i)). In order to assess the effectiveness of this cilia isolation harvesting technique immunofluorescent analysis was performed following cilia removal. Non-coated slides were employed as a negative control and immunofluorescent FITC staining for Arl13b, a protein located solely in the primary cilium, demonstrated that no cilia were isolated in the control (Fig. 2a (ii)). Conversely, when the slides were pre-coated with poly-L-Lysine immunofluorescent FITC staining for Arl13b showed successful isolation of the cilia structure from the cell surface (Fig. 2a (iii)). These findings confirmed that the technique successfully isolated the primary cilia from RPTEC/TERT1 cells.

2.4. NGAL co-localizes with Arl13b in isolated primary cilia

RPTEC/TERT1 cells were differentiated for 10 days post-confluency in 150 mm culture dishes. The isolation technique [23] was employed to isolate the cilia and subsequently the expression of Arl13b (Fig. 2b (i), Fig. 2c (iv)) and NGAL (Fig. 2b (ii), Fig. 2c (ii)) was examined. The merged image indicates that NGAL is co-localised with the ciliary protein Arl13b in the isolated cilia (Fig. 2b (iii), Fig. 2c (iii)).

2.5. Chloral hydrate induces deciliation in RPTEC/TERT1 cells

In order to determine the concentration of chloral hydrate required to induce deciliation without causing cellular damage the human Renal Proximal Tubular cells (RPTEC/TERT1) were cultured in 6 well plates for 10 days post-confluency to allow differentiation before treatment with control medium and increasing concentrations of chloral hydrate (0.5, 1, 2, and 3 mM) for 72 h. Cell viability was assessed using a resazurin assay. Only 3 mM chloral hydrate resulted in a significant reduction in cellular viability compared to the control cells (Fig. 3a (i)). A second assay was performed to assess the cytotoxicity of the different chloral hydrate exposures using an LDH release assay. The cytotoxicity assays supported the findings of the viability assay, with only the 3 mM chloral hydrate exposure inducing a significant increase in cell death, measurable through the increased release of LDH from the cell (Fig. 3a (ii)).

RPTEC/TERT1 cells were then cultured in Millicell® EZ chamber slides for 10 days post-confluency to allow differentiation before treatment with control medium and increasing concentrations of chloral hydrate (0.5, 1, 2, and 3 mM) for 72 h. Immunofluorescence was conducted to determine what effect, if any, the chloral hydrate exposures had on the cell and primary cilia expression. Primary cilia were visualised through the staining of acetylated alpha tubulin, a ciliary protein, with FITC and the nuclei were stained using DAPI. Immunofluorescent microscopy demonstrated the presence of a primary cilium on each cell in the control treatment (Fig. 3b (i)). The immunofluorescent analysis indicated that the number of cilia present on the cells decreased in conjunction with increasing concentrations of chloral hydrate (Fig. 3b (ii–vi)), with concentrations in excess of 1 mM resulting in a significant loss of primary cilia from the cells (Fig. 3b (vi)). Based on this data it was determined the a 2 mM chloral hydrate concentration induced significant deciliation in the RPTEC/TERT1 cells, but this concentration would be sub-cytotoxic for the cells and would not induce cell damage as evidenced by the viability and cytotoxicity assays.

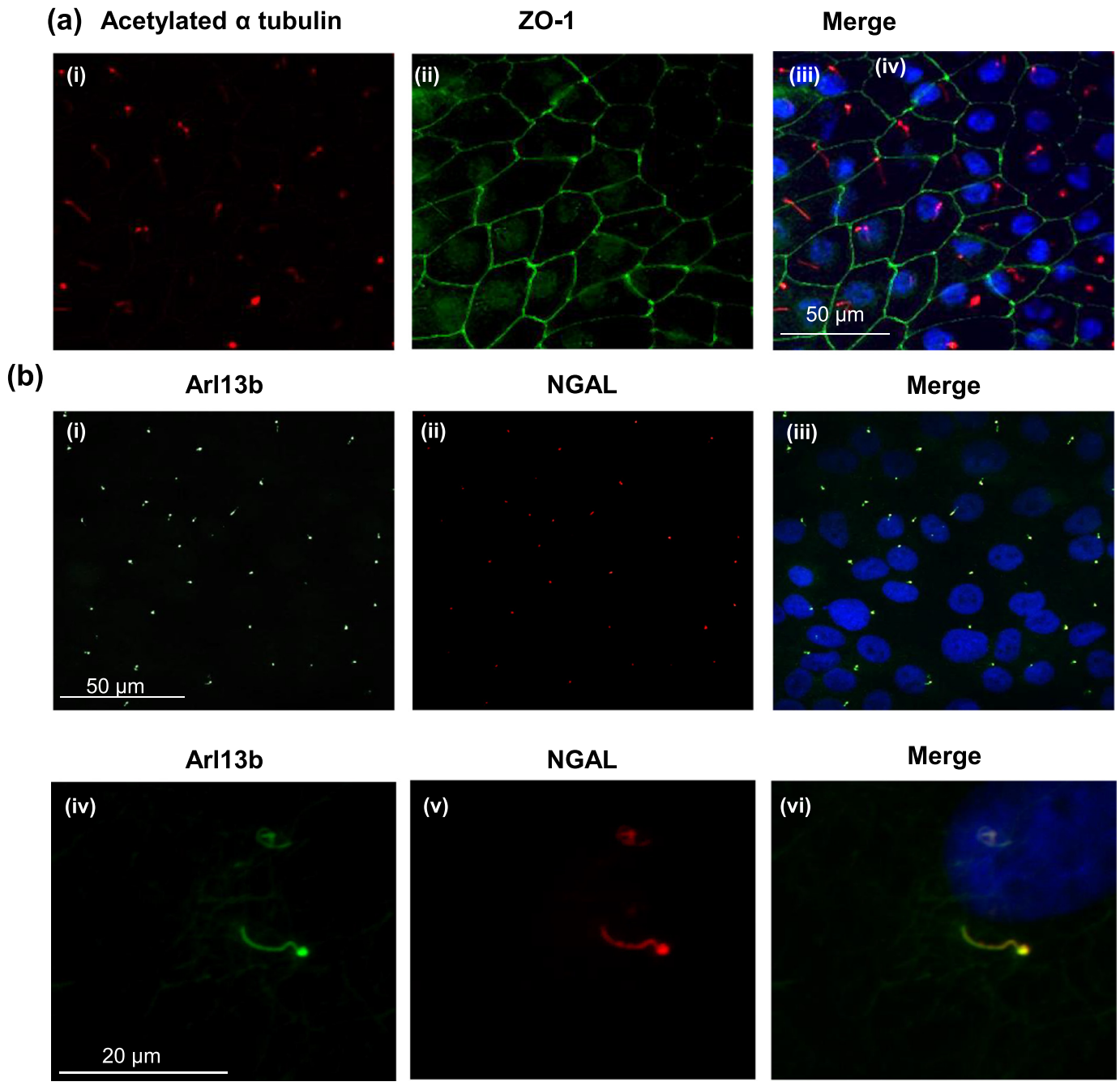


Fig. 1. Confluent RPTEC/TERT1 cells express a primary cilium.
RPTEC/TERT1 cells were cultured for 10 days following confluency. (a) Immunofluorescent labelling of (a i) acetylated α-tubulin showing multiple cilia, (a ii) Zona Occludens (ZO-1), (a iii) Merged acetylated α-tubulin, ZO-1 and DAPI staining and (iv) at 40× magnification. (b) Immunofluorescent labelling of control cells with nuclei stained with DAPI (blue), (b i) Arl13b, a specific ciliary protein, stained with FITC (green), (b ii) NGAL stained with Texas Red (red), (b iii) merged image of NGAL, Arl13b and nuclei, and (b iv–vi) zoomed images of the previous images.

2.6. Mechanism of primary cilia loss in RPTEC/TERT1 cells

Human Renal Proximal Tubular cells (RPTEC/TERT1) were cultured in Millicell® EZ chamber slides for 10 days post-confluency to allow differentiation before treatment with control medium, 10% serum, or 2 mM chloral hydrate for 72 h. Immunofluorescence was conducted to determine what effect, if any, the treatments had on the cell and primary cilia expression. Epithelial cell borders were visualised through the staining of ZO-1 with FITC, the cilia were visualised using staining of acetylated alpha tubulin, another ciliary protein, with Texas red and the nuclei were stained using DAPI. As previously stated, Arl13b was selected due as it is a highly specific ciliary marker protein. The analysis showed that in the healthy control cells, the cells displayed the typical epithelial morphology, with a tight monolayer of closely packed cobblestone-like cells. Each individual cell expressed a primary cilium (Fig. 4a (i)). Exposure to 10% serum greatly affected the typical RPTEC/TERT1 morphology, with disruptions in the normal expression of the tight junction protein ZO-1 evident. Additionally, changes to the primary cilia are evident with the cilia shortened or missing on most cells, with re-distribution of the ciliary protein acetylated alpha tubulin within the cell cytoplasm (Fig. 4a (ii)). Following exposure to 2 mM chloral hydrate resulted in a complete reduction in cilia presence and

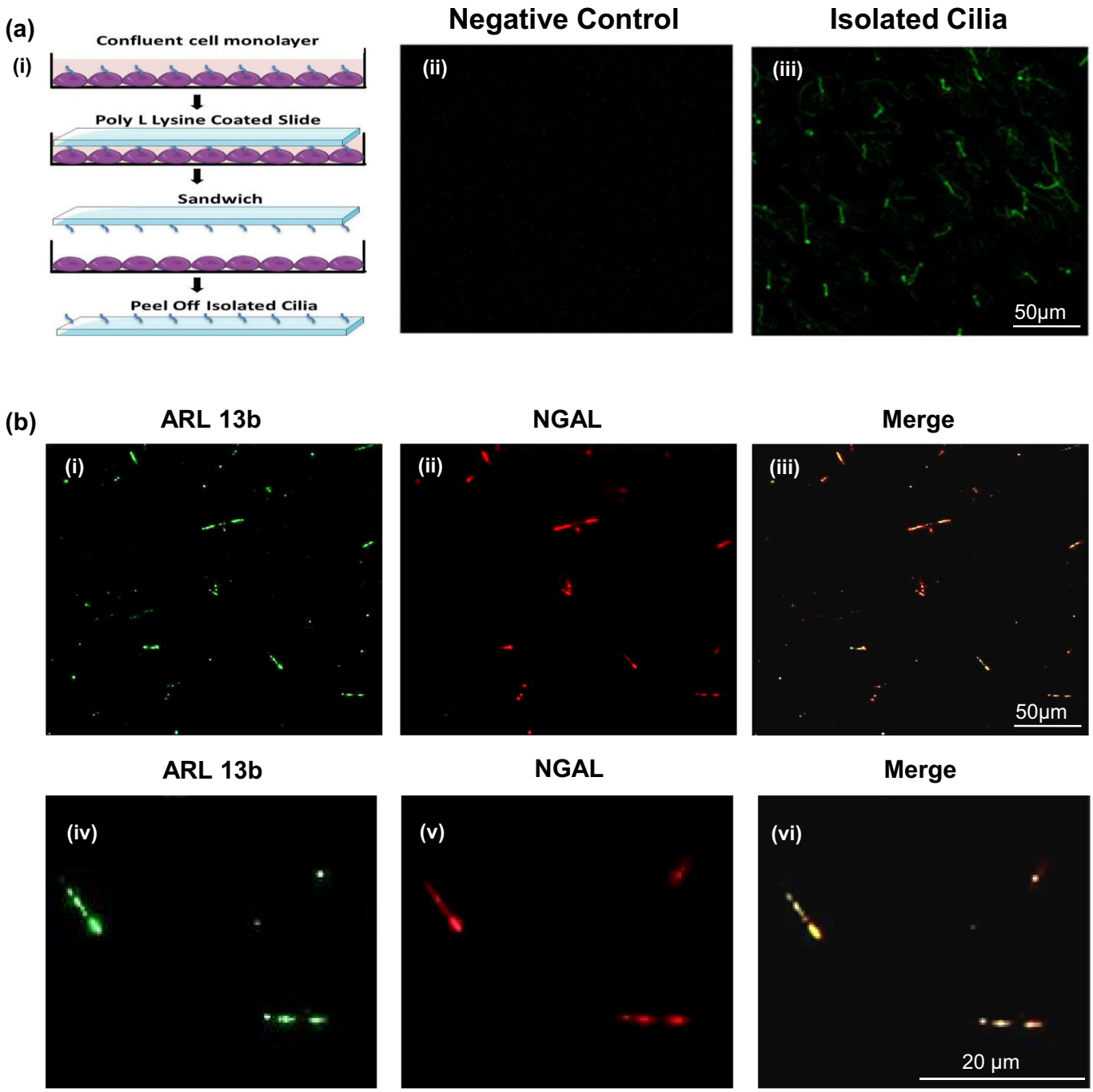


Fig. 2. Deciliation of confluent RPTEC/TERT1 cells.
RPTEC/TERT1 cells were cultured for 10 days following confluency. (a i) Deciliation was performed using the peel-off method. (a ii) Normal non-coated cover slips and (a ii) Poly L lysine coated cover slips were used to remove the primary cilia from the cells and were subsequently analysed using immunofluorescence. Immunofluorescent labeling of acetylated α-tubulin in (a ii) the non-coated slide and (a iii) on the poly-L lysine coated cover slip, showing multiple cilia. (b) Deciliation of RPTEC/TERT1 cells using the peel off method. Slides were immunoflourescently labelled for (i) Arl13b, (ii) NGAL and (iii) merged image of Arl13b and NGAL staining. Also shown are magnified images for (iv) Arl13b, (v) NGAL and (vi) merged image of Arl13b and NGAL staining.

length as well as alterations to the typical morphology of the RPTEC/TERT1 cells, which appear to take on a more fibrotic phenotype, becoming elongated and spindle-like (Fig. 4a (iii)).

Image J was utilised to analyse the images by morphometric analysis for the presence of ciliated cells, with the results showing a clear decrease in the number of ciliated cells following treatment with 10% serum (83%) and a further decrease in ciliated cell numbers following chloral hydrate treatment (56%) (Fig. 4b (i)). Additionally, ImageJ was employed to analyse cilia length in response to treatment, demonstrating both treatments when compared to control (approximately 5 μm in length) resulted in a decrease in cilia length, with the serum treated cells presenting with the shortest cilia (Fig. 4b (ii)).

However, considering the shortened cilia observed following exposure to 10% serum, coupled with the redistribution of Arl13b within the cell as evidenced by immunofluorescence, it can be proposed that serum results in resorption of the cilia as opposed to deciliation. Conversely, 2 mM chloral hydrate resulted in shortening of the cilia, without any evidence of redistribution of the ciliary protein Arl13b in

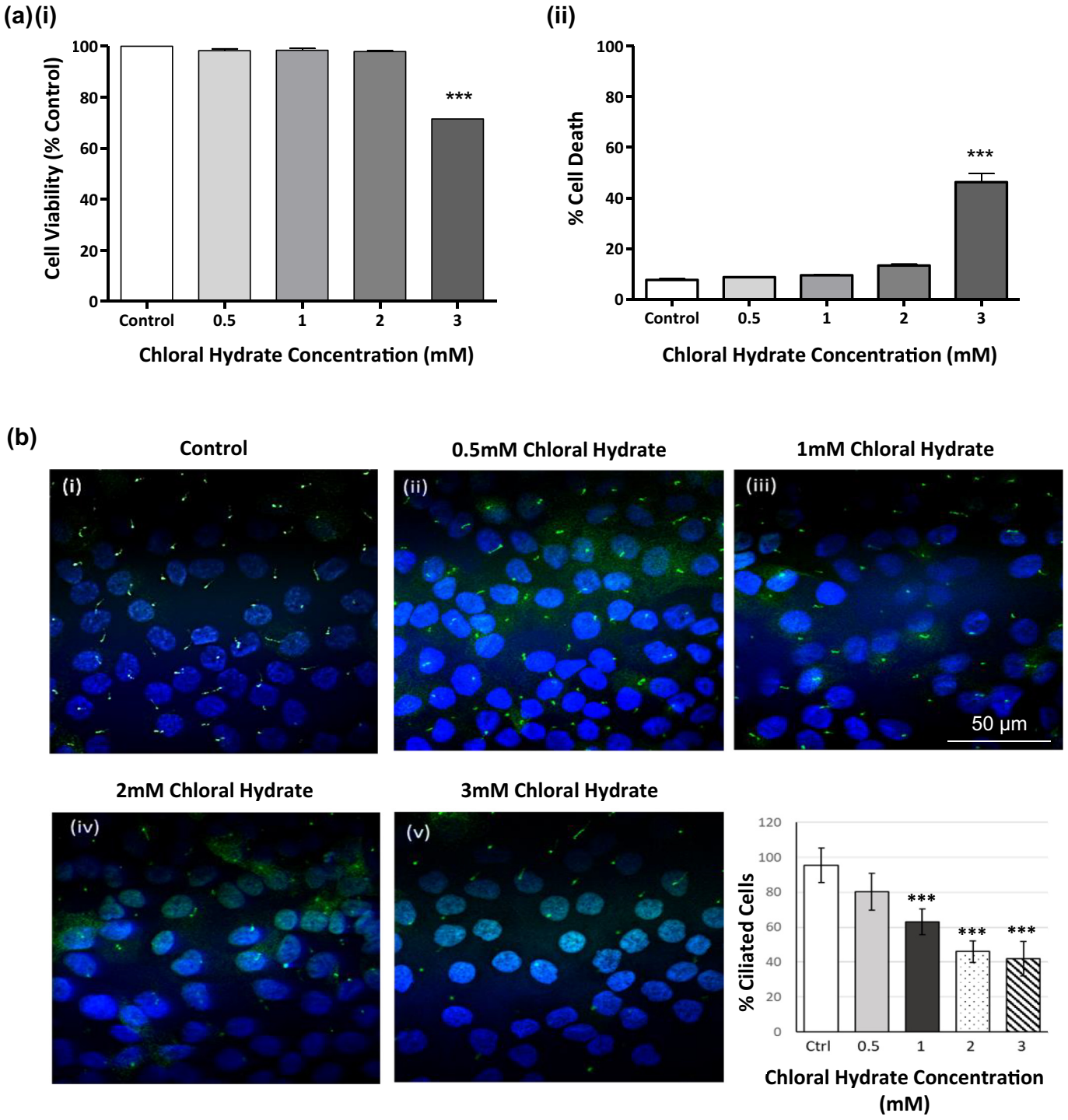


Fig. 3. Deciliation of confluent RPTEC/TERT1 cells using chloral hydrate.
(a) RPTEC/TERT1 cells were cultured in 6 well plates for 10 days following confluency. Cells were treated with control medium or increasing concentrations of chloral hydrate for 72 h. The effects of increasing concentrations of chloral hydrate on (a) (i) cell viability and (ii) cytotoxicity was assessed (n = 3). (b) The effects of increasing concentrations of chloral hydrate on the expression of the primary cilia was investigated by immunofluorescence. Immunofluorescent labelling shows the merged image of acetylated alpha tubulin (FITC, green) and DAPI nuclei staining for (b i) control, (b ii) 0.5 mM chloral hydrate, (b iii) 1 mM Chloral hydrate, (b iv) 2 mM Chloral hydrate and (b v) 3 mM chloral hydrate. (b vi) Following treatment immunofluorescent labelling of acetylated alpha tubulin allowed determination of (b i) percentage of ciliated cells (n = 3).

the cellular cytoplasm, indicating the ciliary loss occurs through deciliation [24–27].

2.7. Cellular loss of NGAL coincides with removal of the primary cilia in RPTEC/TERT1 cells

To determine whether the loss of the primary cilium corresponded with a loss of NGAL from the cell, regardless of the mechanism, NGAL and Arl13b were further examined using western blot. Cells were grown

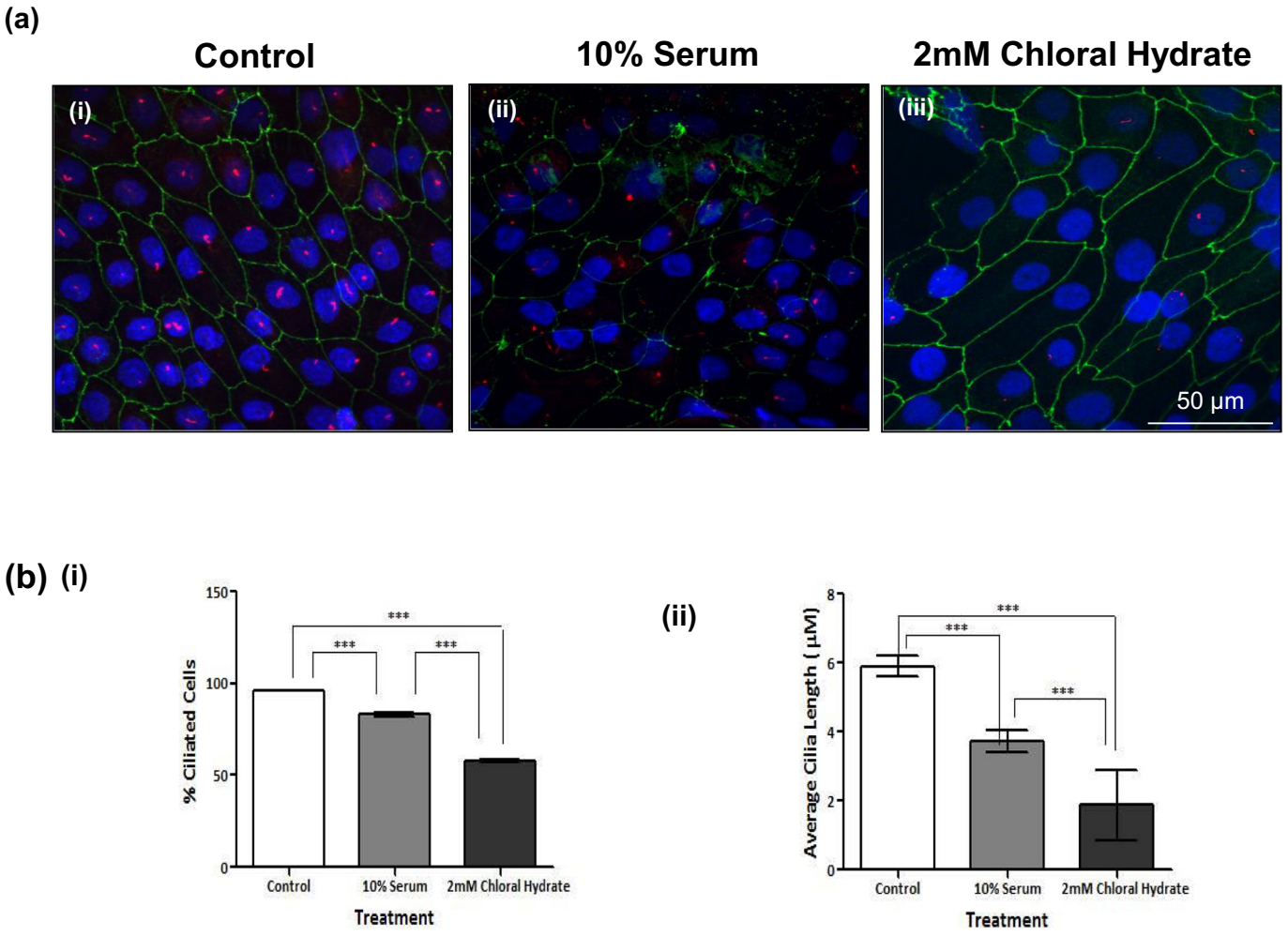


Fig. 4. Deciliation of confluent RPTEC/TERT1 cells using chloral hydrate.
RPTEC/TERT1 cells were cultured for 10 days following confluency. (a) Cells were treated with control medium, 2 mM chloral hydrate or 10% serum for 72 h. (a) Immunofluorescent labelling showing merged image of ZO-1 (FITC, green), acetylated alpha tubulin (Texas red) and DAPI nuclei staining for (a i) control, (a ii) chloral hydrate and (a iii) 10% serum. (b) Following treatment immunofluorescent labelling of ZO-1 and acetylated alpha tubulin allowed determination of (b i) percentage of ciliated cells (n = 3) and (b ii) average cilia length (µM) (n = 3).

to confluency and maintained for 10 days before treatment with 10% serum or 2 mM chloral hydrate for 72 h. Western blot analysis was conducted to analyse the whole cell lysates and supernatants for the presence of NGAL and the ciliary proteins Arl13b and acetylated alpha tubulin. Lysates from RPTEC/TERT1 cells treated with 10% serum resulted in a slight decrease in NGAL and acetylated alpha tubulin compared to control ($p < 0.01$ and $p < 0.001$ respectively). The 10% serum exposure resulted in a significant increase in Arl13b in the lysate, suggesting the ciliary structure has been resorbed. Treatment with chloral hydrate however resulted in a statistically significant loss of both NGAL, Arl13b and acetylated alpha tubulin in the lysates (Fig. 5a (i, ii, iii)). Focusing on the supernatants, both the control and 10% serum samples showed similar low levels of NGAL, Arl13b and acetylated alpha tubulin. Conversely chloral hydrate shows a distinct increase in both NGAL, Arl13b and acetylated alpha tubulin in the supernatants (Fig. 5b (i, ii, iii)).

2.8. NGAL co-precipitates with Arl13b in human urine samples

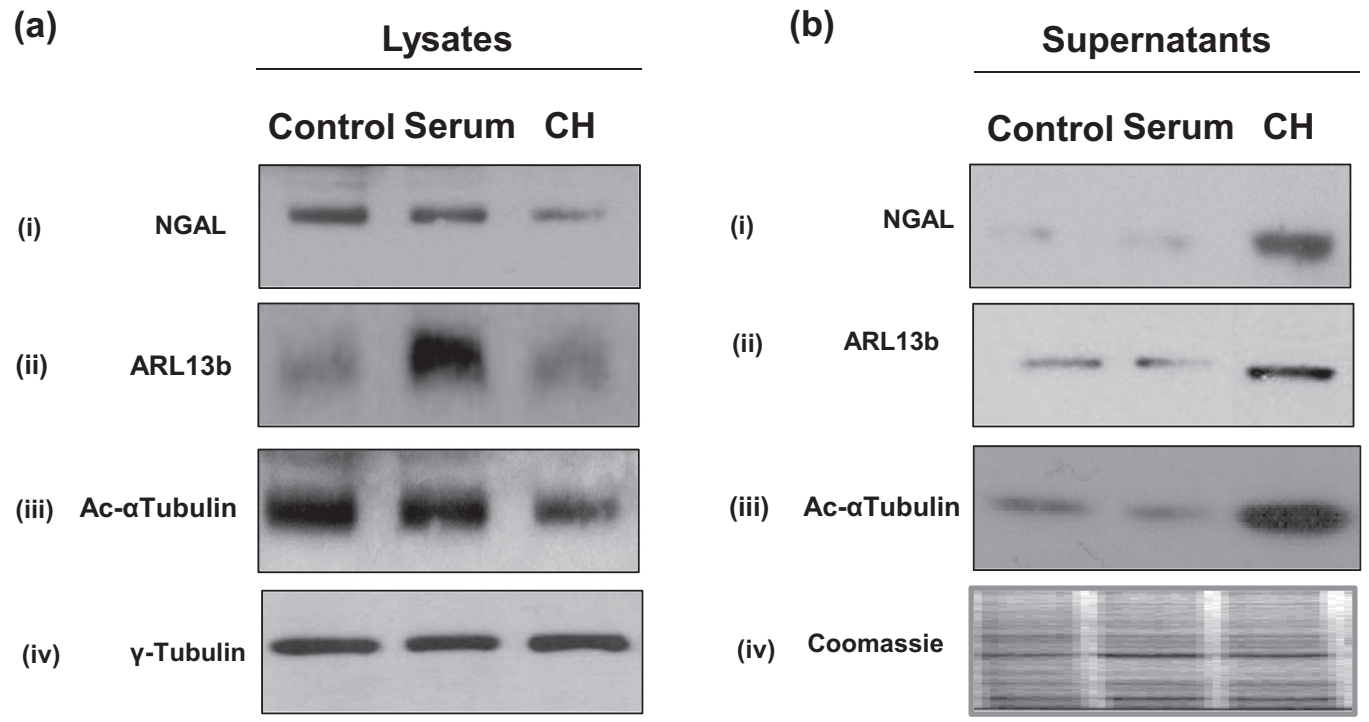
In order to determine whether NGAL co-localised with ciliary proteins such as Arl13b co-immunoprecipitation experiments of Arl13b and NGAL were performed in urine samples from a Chronic Allograft Nephropathy (CAN) cohort. Dynabeads® were used to precipitate Arl13b from the samples and western blot analysis for Arl13b showed the sample was successfully precipitated (Fig. 6a). Additionally, western blot analysis also detected NGAL in the co-immunoprecipitation samples, indicating that NGAL co-localizes with the ciliary protein Arl13b (Fig. 6a).

2.9. NGAL and Arl13b urinary expression increases with the severity of CAN

Aliquots of the urinary samples from the clinical cohort were pooled according to their BANFF grading (Supplementary Table 1) and western blot analysis was performed to assess the expression of NGAL and Arl13b in the clinical cohort. Urinary expression of both NGAL (Fig. 6b (i)) and Arl13b (Fig. 6b (ii)) were shown to increase as the disease progressed. The expression of NGAL was shown to be significantly increased at BANFF stage 3 (severe CAN) (Fig. 6b (iv)), whilst Arl13b was significantly increased at both BANFF stage 2 (moderate CAN) and BANFF stage 3 (Severe CAN) (Fig. 6b (v)).

2.10. Clinical relevance of ciliary loss - quantification of NGAL in chronic allograft nephropathy clinical cohort

In collaboration with Beaumont hospital, urine samples from CAN



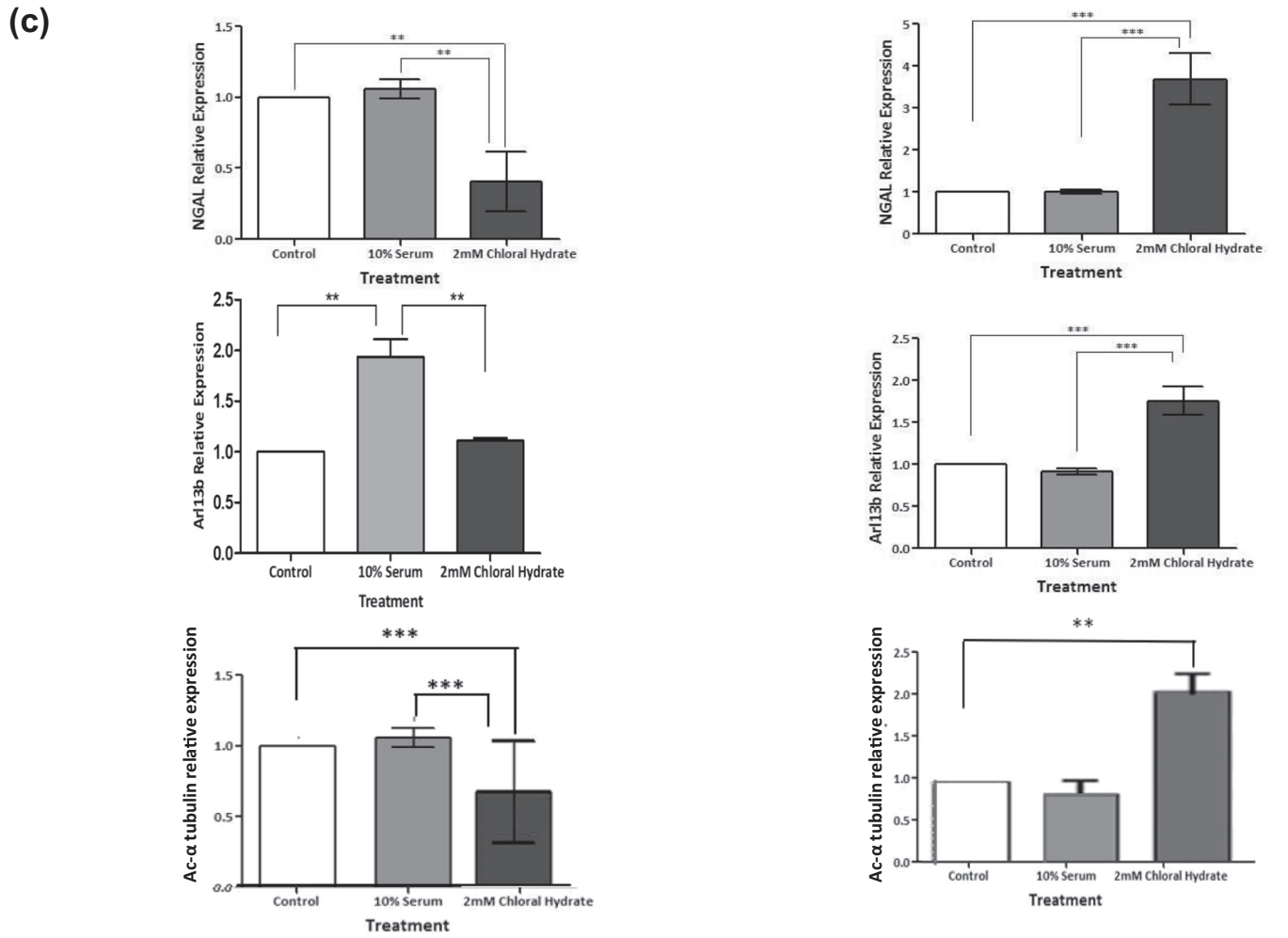


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Fig. 5. Effect of treatments on cilia in RPTEC/TERT1 cells.
RPTEC/TERT1 cells were cultured for 10 days following confluency before treatment with control medium, 10% serum or 2 μM Chloral Hydrate. (a) Whole cells lysates were prepared using RIPA buffer and analysed by western blot for expression of (a i) NGAL, (a ii) Arl13b, (a iii) acetylated alpha tubulin and (iv) gamma tubulin which was employed as a loading control. (b) Following treatments supernatants were collected, even amounts of protein was loaded onto the gels using strataclean resin and probed for (b i) NGAL, (b ii) Arl13b and (b iii) acetylated alpha tubulin. Coomassie staining of an SDS-PAGE gel was employed as a loading control. (c) Densitometry is shown for (i) NGAL expression in whole cell lysates, (ii) Arl13b expression in whole cell lysates, (iii) NGAL expression in supernatants and (iv) Arl13b expression in supernatants.

patients were analysed for the presence of NGAL. NGAL protein expression was shown to increase significantly as the BANFF grade increased, indicating A statistically significant difference was detected between control and CAN urine samples based on the raw NGAL data ($p < 0.01$) (Fig. 6c (i)). The mean NGAL concentration for the control group was 189.01 pg/ml compared to 708.3 pg/ml for the CAN patient group. When the individual Control and CAN patient concentrations were normalised to urinary creatinine (uCr) values, the significance between the two groups increased ($p < 0.001$) (Fig. 6c (ii)). The mean normalised NGAL concentration for control samples was 0.0235 pg/mM compared to 0.1191 pg/mM for CAN samples (Fig. 6c (ii)). Further analysis of the ELISA data in relation to the clinical data, in particular the urinary creatine values (Supplementary Table 1) shows that the detectable concentration of NGAL in the urine increases as the disease severity worsens, denoted by increasing BANFF grades (Table 1).

3. Discussion

Due to its near-ubiquitous expression on almost all cells in the human body, the primary cilium has moved from being regarded as a vestigial artefact to a position of considerable importance in biomedicine. Evidence now supports the role of the primary cilium in the regulation of a large number of cellular processes, highlighting how crucial the primary cilium is to normal physiological function. It is now widely acknowledged that cilia, whether motile or immotile, are integral to an ever-growing list of cellular processes including chemosensation, mechanosensation, phototransduction and left-right patterning in development [22,28,29].

In addition to the numerous roles played by the primary cilium in maintaining normal epithelial function, the cilium is now gaining attention with regards to potential roles in disease, particularly kidney disease. The physiological importance of the central cilium is highlighted by the growing list of disorders where the primary aetiology is rooted in abnormal ciliary function, termed “ciliopathies” [12,22] Primary cilia play a central role in epithelial function within the kidney, both normal healthy kidneys and diseased kidneys [11,30,31]. Therefore, the presence of the primary cilium in RPTEC/TERT1 cells functions an indicator of correct epithelial functions and cellular differentiation. Ciliary loss from the renal proximal tubule cells would indicate renal damage. Therefore, the primary cilium represents a potentially rich source of biomarkers of renal injury. In 2007 it was reported that Kidney Injury Marker 1 (KIM1), a renal biomarker of acute kidney injury (AKI), chronic kidney disease (CKD) and chronic allograft nephropathy (CAN), was expressed in primary cilia [18,32]. Therefore, it is possible that the expression of other well-known renal injury biomarkers, and currently unidentified renal biomarkers, may be linked to the primary cilium.

It has been documented that primary cilium loss may occur through two distinct mechanisms; during resorption the primary cilium is reabsorbed into the cell body, while during deciliation (or autotomy) the entire organelle is shed from the surface of the cell [33,34]. Removal of the primary cilium through reabsorption occurs as cells progress through the cell cycle. Indeed removal of the cilium is a necessary event for mitosis to occur as the mother centrioles which nucleate the primary cilia also contribute to formation of the centrosome, the microtubule organizing centre responsible for arranging the mitotic spindles during mitosis [35]. Studies have suggested that resorption or retraction of the primary cilia can be induced by serum stimulation, which causes the disassembly of the primary cilia by destabilizing the microtubules [36–38]. Ciliary shedding on the other hand may be a response to exposure to toxic stimuli [34]. Studies have shown that exposure to chloral hydrate induces deciliation by breaking the junction between the cilium and the basal body and is frequently employed as a positive control for ciliary autonomy or the “chopping off” of the primary cilium [26,34,39–41]. In this study, we verified that 10% serum exposure results in resorption of the cilia, evidenced immunofluorescently by the re-distribution of the ciliary proteins within the cytosol. In contrast, chloral hydrate resulted in almost complete loss of the protein from the cell, with no redistribution of the ciliary proteins, indicating that the cilium has been autotomized or chopped off. Additionally, successful implementation of a deciliation isolation method [23] was undertaken and utilised during this study to successfully remove primary cilia from the confluent, differentiated RPTEC/TERT1 cells, as evidenced by Arl13b staining of the cilia on the pull off slide.

Immunofluorescence for the localisation of NGAL in the human renal cells demonstrated that the protein is expressed exclusively within the primary cilia, co-localizing with ARL13b, a widely accepted ciliary protein. This is not a new concept, another popular renal biomarker KIM-1 was shown to be expressed in the primary cilium [32]. Using the previously described isolation method [23] and immunofluorescent staining it was demonstrated that NGAL is lost with the cilia structure, co-localizing with the accepted ciliary biomarker Arl13b. This is a novel observation which offers important insights regarding the mechanism underlying increased urinary NGAL in renal disease. Our results suggest that the cilia of renal epithelial cells are shed in direct response to cellular injury and that this contributes to the biomarker profile in the urine of patients.

To further investigate whether NGAL levels correlated with the loss of cilia, western blot analysis was employed to analyse the effects of resorption and deciliation on the expression of NGAL and Arl13b in the lysates and supernatants of RPTEC/TERT1 cells treated with two established cilia loss inducing agents. The results demonstrate that 10% serum, which results in ciliary resorption, caused only a slight decrease in NGAL and Arl13b in whole cell lysates compared to control. Western Blot analysis of the supernatants indicated low levels of both ARL13b and NGAL. This suggests that the serum is causing the cilia to be resorbed into the cell, which in turn is re-distributing the proteins into the cytosol which is then detectable in the lysates. Conversely chloral hydrate, which we have shown induces deciliation, demonstrated low cellular levels of the proteins compared to the control, but the concentrations of both proteins were greatly increased in the supernatants. This suggests that NGAL is lost from the cell in conjunction with cilia loss.

To investigate the clinical relevance of this observation immunoprecipitation of clinical CAN urine samples was utilised to investigate whether NGAL could be co-immunoprecipitated with the ciliary protein Arl13b. NGAL was indeed detected by western blot when Arl13b was pulled down in CAN urine samples. This suggests that the increased levels of NGAL observed in Chronic Allograft Nephropathy, and in a wider kidney disease context, correlate with ciliary loss. Quantification of NGAL in urine samples collected as part of the described cohort showed statistically increased urinary NGAL concentrations in CAN patients ($p < 0.001$). NGAL is already widely accepted as a biomarker for various renal diseases and the findings of this study

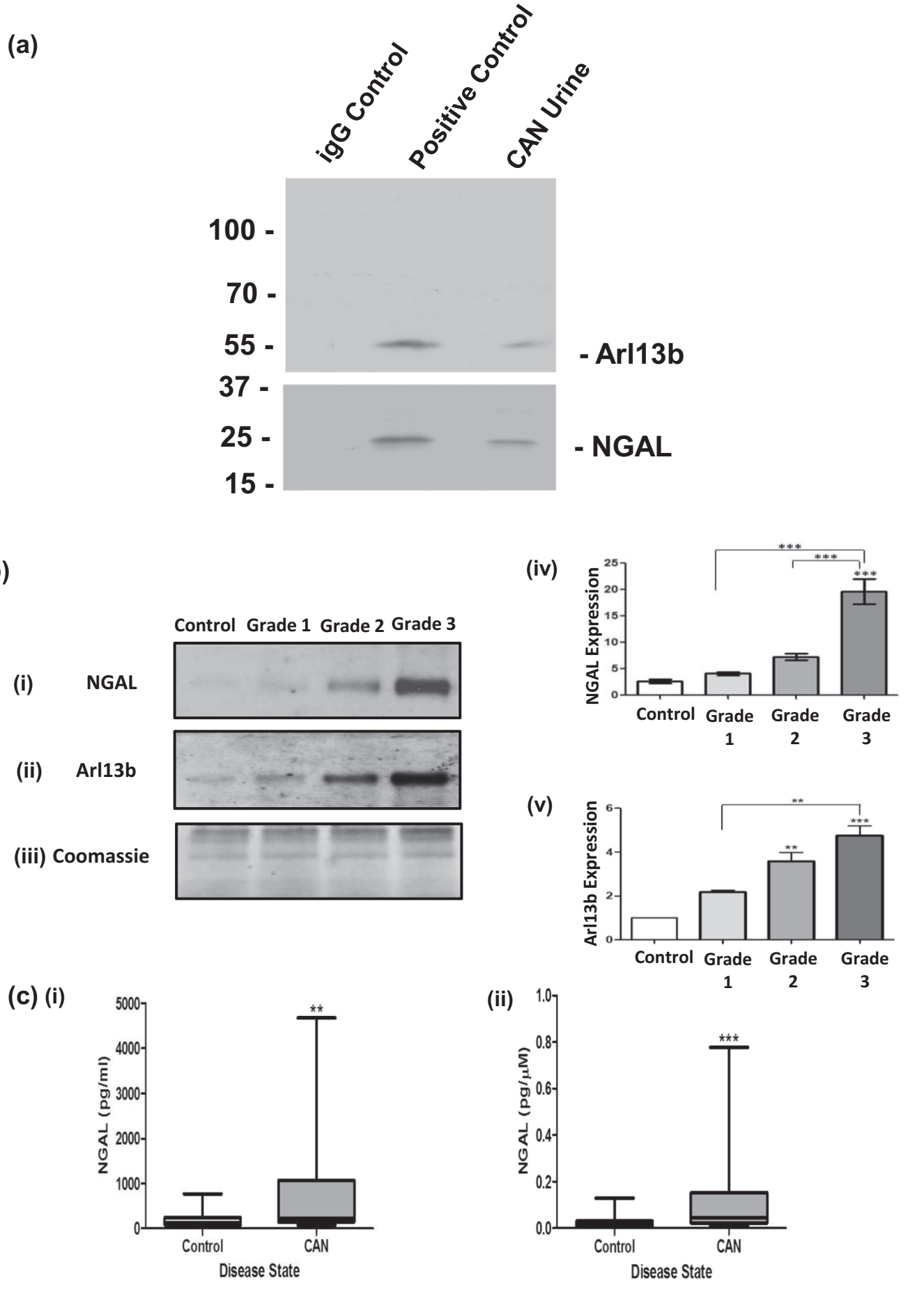

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Fig. 6. Detection of NGAL in clinical urine samples.
Co-immunoprecipitation was carried out on urine samples using Dynabeads coated with 10 μg Arl13b and the precipitated protein eluted and resuspended in sample buffer before running on an SDS-PAGE gel along with an NGAL control for western blot analysis. The membranes were then probed for (a i) Arl13b expression and (a ii) NGAL expression. (b) Urine samples were assessed by western blot probing with monoclonal antibodies against (b i) NGAL and (b ii) Arl13b. Coomassie staining was employed as a loading control (c) Densitometry was performed NGAL was quantified in a Chronic Allograft Nephropathy (CAN) clinical urine samples by ELISA. (c i) raw data expressed as picograms per milliliter (pg/ml) and (c ii) Normalised to individual patient urinary creatinine levels (uCr).

confirm that NGAL may have a use as a biomarker for the development and progression of CAN in transplant patients.

Using a combination of proteomic analysis by western blot and immunofluorescent labelling this study has shown that the primary cilium is a potential source of biomarkers of kidney disease. The study also demonstrated that NGAL is in fact a novel ciliary protein, co-localizing with known ciliary proteins. This study establishes a link between the increase in NGAL in urine samples following renal injury and the loss of cilia from the renal epithelial cells. Perhaps more importantly, the study also demonstrated that NGAL expression in supernatants and urine samples correlated with Arl13b expression. This offers a novel explanation for the mechanism by which NGAL accumulates in urine during kidney disease. Further analysis of the ciliary proteome could yield novel early biomarkers of renal injury and disease.

4. Concise methods

4.1. Cell culture

The human RPTEC/TERT1 renal proximal tubular epithelial cell line [Evercyte, Vienna, Austria] was cultured in low glucose (5.5 mM) Dulbecco's Modified Eagle Medium/Nutrient mix F12 (DMEM/F12) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml selenite, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 50 units per ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine culture media [Gibco Life Technologies CA USA] in a 5% CO_2 humidified atmosphere at 37 °C [22] Cells were maintained for 10 days post-confluency to allow for epithelial polarisation, differentiation and cilia formation. Spent medium was replaced every 48 h with pre-warmed fresh culture medium.

4.2. Treatments

RPTEC/TERT1 cells were treated with control medium, 2 mM chloral hydrate or 10% serum. 2 mM chloral hydrate was prepared fresh for each experiment with 0.1 g of chloral hydrate [Sigma-Aldrich USA] dissolved in 50 ml complete media and filter sterilised prior to use. The 10% serum solution was prepared by adding 50 ml foetal calf serum [Gibco Life Technologies] to 450 ml complete medium.

4.3. Immunofluorescence

RPTEC/TERT1 cells were cultured in 8-well Millicell EZ chamber slides [Millipore, MA, USA] for 10 days following confluency then treated with the appropriate compound for 72 h. Cells were then washed once with ice cold PBS and fixed with 3.7% paraformaldehyde for 20 min at room temperature. The cells were then permeabilized with 0.2% (v/v) Triton-X 100 in PBS for 20 min. Cells were then washed 3 times in PBS with gentle agitation. Non-specific binding was reduced by blocking in 0.5% (w/v) BSA/PBS for 1 h at room temperature. The cells were then incubated in 300 μl per well of the primary antibody for 1 h. Antibodies employed include 1:200 Arl13b [ProteinTech, IL, USA], 1:200 acetylated α-tubulin [Sigma Aldrich] and 1:500 NGAL [Acris Antibodies GmbH, Germany] were used for immunofluorescent staining.

Following this, cells were washed 3 times with PBS and gentle agitation. Subsequently, the cells were then incubated with 300 μl per well of the secondary antibody (1:200 dilution of anti-rabbit IgG antibody, anti-mouse IgG or donkey-anti goat antibody, either FITC or Texas red [AlexaFluor, ThermoFisher] prepared in 0.5% (w/v) BSA/PBS) in the dark for 1 h. Cells were then washed 3 times with PBS. The chambers were then removed and 15 μl ProLong® Gold antifade reagent with DAPI [Molecular Probes] before a coverslip was mounted. The DAPI acts as a nuclear stain. Immunofluorescence was visualised using a Zeiss LSM 510 Meta confocal laser scanning microscope and LSM5 image acquisition software.

4.4. Poly-L-Lysine slide preparation

Poly-L-Lysine slides were prepared by first cleaning glass slides in acid alcohol (1% HCL in 70% ethanol) on an orbital shaker for 2 h before rinsing the slides with four double distilled water washes to remove all traces of ethanol. A poly-L-Lysine solution was prepared by diluting the stock solution 1:10 in deionized water. The clean glass slides were placed in a rack and submerged in the diluted poly-L-Lysine solution for 5 min before the slides were drained and the slides were dried in a 60 °C oven for 1 h.

4.5. Cilia isolation

Cilia isolation was conducted in a 150 mm confluent differentiated plate of RPTEC/TERT1 cells using a previously described sandwich technique [23]. Briefly, a poly-L-lysine coated coverslip was placed on the cell monolayer and the culture media was aspirated off whilst applying light pressure to the top of the coverslip by pushing down slightly with a finger for 20 s. The coverslip is then removed from the cell monolayer. The samples were then fixed with a 4% formaldehyde solution before further analysis by Immunofluorescence.

4.6. Morphometric analysis

Cilia length was analysed by generating z stack confocal images in Image J (National Institutes of Health) and the frequency and length of the cilia were measured manually using the Image J Segmented line tool. Statistical analysis of the Image J data was performed using a one-

Table 1
Fractional excretion data for NGAL: The data is expressed as raw values (ng/ml) or normalised to urinary creatinine (pg/ml).

	Unnormalized values			Normalised to urinary creatinine		
	Median (ng/ml)	Range (ng/ml)	Significance	Median (pg/ml)	Range (pg/ml)	Significance
Control	0.1131	0.007916–0.5599		0.001628	6.249e-005–0.04254	
BANFF Grade 1	0.1632	0.1578–0.1685		0.006192	0.004393–0.007992	
BANFF Grade 2	0.4847	0.4131–0.5563		0.01402	0.01129–0.06822	$p < 0.01$
BANFF Grade 3	0.5112	0.1061–1.855	$p < 0.001$	0.02273	0.005019–0.07228	$p < 0.001$

way ANOVA with an accompanying Tukey's *post hoc* test performing intergroup comparisons. Values are expressed as mean ± SEM.

4.7. Western blot analysis

Whole cell lysates were extracted using RIPA buffer containing a protease inhibitor cocktail [Sigma Aldrich]. Protein concentration was measured by BCA Protein Assay (Pierce) according to the manufacturer's instructions. Twenty micrograms of protein were boiled in loading buffer and separated by electrophoresis on a 12% polyacrylamide gel. Gels were transferred to a nitrocellulose membrane at 80 mA for 1 h 40 min using a semi-dry transfer system [Semi-Phor™ Hoeffer Scientific Instruments]. Following ponceau staining to verify transfer the membrane was blocked in 5% milk powder in TBS-T for 1 h at room temperature. The membrane was then incubated overnight at 4 °C in antibodies against NGAL [Acris Antibodies GmbH], Arl13b [Cell Signalling Technologies], acetylated α-tubulin [Sigma Aldrich] and β-actin [Abcam]. Membranes were then incubated in secondary antibody (1:200) for 1 h at room temperature. A horseradish peroxidase-linked secondary antibody was used. Protein bands were visualised using chemiluminescence [Pierce]. Statistical analysis was carried out using ANOVA and Bonferroni post testing.

4.8. Resazurin assay - cell viability assay

Cells were grown to confluency on Costar 6-well plates. The resazurin stock (20× stock, 880 mM) was prewarmed to 37 °C and diluted 1:20 in DMEM/F12 supplement medium to a final volume sufficient for the entire plate. Following treatment, the media was removed from the wells and replaced with 2 ml of the prediluted resazurin stock. Cells were then incubated at 37 °C for 90 min to allow the resazurin to be metabolised in the culture media by viable cells to its reduced state. Fluorescence was read at 545 nm on a Wallac Victor-V multi-well plate reader. The amount of fluorescence detected is proportional to the percentage of viable cells.

4.9. Lactose dehydrogenase release (LDH) assay - cytotoxicity assay

An LDH cytotoxicity assay kit was employed to measure cell death (Roche). The 1× LDH assay cofactor was prepared by adding 25 ml of tissue culture grade water to bottle of lyophilized cofactors. The treated cells were removed from incubator into a laminar flow hood. Aliquots of culture medium were transferred to Eppendorf tubes and were centrifuged at 250G for 4 min. The aliquots were transferred into the wells of a clean flat-bottomed plate for enzymatic analysis. Lactate dehydrogenase assay mixture was prepared by mixing equal amounts of LDH assay substrate, cofactor and dye solutions. The assay mixture was added in an equal amount to 2× the volume of medium to be assayed to each sample (100 μl LDH mix to 50 μl sample). The plate was covered with aluminium foil to protect from light and incubated at RT for 30 min. The reaction was terminated by adding a tenth volume of 1 N HCL to each well. Absorbencies were read spectrophotometrically (Wavelength = 490 nm). Background absorbance of the multiwell plate was measured at 690 nm and subtracted from the primary wavelength measurement. Cells treated with 1% Triton X was employed as the 100% cytotoxicity control for the experiment.

4.10. Ciliary fraction

RPTEC/TERT1 cells were cultured on 150 cm culture plates for 10 days post confluency. Cell supernatant was collected in a 50 ml conical tube, the cells were washed briefly with 5 ml of PBS with gently rocking to detach cilia, before also adding this PBS solution to the conical tube. The tubes were centrifuged in a Beckman J6 centrifuge at 1000*g* for 10 min at 4 °C. The position of the pellet containing cellular debris was marked with a felt tip pen, transferring the supernatant to a clean ultracentrifuge tube, taking care not to disturb the pellet. The pellet and cell debris left over was discarded. The new tube was centrifuged at 40,000*g* for 30 min at 4 °C, before marking the pellet position and removing the supernatant. The pellet was resuspended in 20 μl of resuspension buffer [200 μl 1 M Tris-HCL pH 8, 37.2 mg KCL, 4.4 mg $MgSO_4$, 1.5 mg DTT, 10 μl 0.5 M EDTA pH 8 and brought to final volume of 10 ml with H_2O] by gentle pipetting at which point the sample was used for further analysis or frozen at −80 °C.

4.11. Study population and sample collection

The study design was previously reported by Johnston et al. [18,42]. Briefly, approval was gained from the Beaumont hospital ethics in medical research committee for studies involving human subjects. Midstream urine samples were obtained from a cohort of renal transplant patients who were attending the renal transplant clinic at Beaumont Hospital, Dublin, between July 2004 and April 2005 and who complied with the inclusion and exclusion criteria [18,42] and provided informed consent. The “disease” group included 34 renal transplant patients with histologically proven CAN (according to Banff 1997 criteria) [18] and the “control” group included 36 renal transplant patients with normal renal function (serum creatinine < 140 μMol/L; glomerular filtration rate > 50 mL/min) > 1 year after transplantation. Additionally, patients diagnosed with CAN as a result of a renal transplant biopsy, between 1987 and 2005, (according to the Banff 1997 criteria) were invited to participate once the inclusion criteria were met [18,42]. Clinical and historical data were documented for each patient, including age, gender, and immunosuppression regimen and creatinine values [18]. Clinical data such as BANFF classifications, creatinine levels, glomerular filtration rates (GFR), creatinine clearance and urinary creatinine levels are shown in Supplementary Table 1.

4.12. Immunoprecipitation

50 μl of Dynabeads® Protein G was transferred to a tube and the tube was placed on the magnetic holder to separate the beads from the solution and the supernatant was aspirated off. 10 μg of the antibody Arl13b, diluted in 200Ul PBS/Tween 20, was added to the beads and incubated with rotation for 10 min at room temperature. The supernatant was removed using the magnetic holder and then the bead-antibody complex was re-suspended in 200 μl PBS/Tween 20. The tube was placed in the magnetic holder and the supernatant was removed. After removing the tube from the magnetic holder 500 μl of the urine sample was added to the Dynabeads-antibody complex and gently pipetted to re- suspend the beads. Following incubation with rotation for 10 min at room temperature to allow the antigen to bind to the Dynabeads-antibody complex the tube was placed on the magnet and the supernatant was removed. The Dynabeads-antibody-antigen complex was washed three times with 200 μl PBS, using the magnet to pull down the beads before removing the supernatant. The Dynabead-antibody-antigen complex was re-suspended in 100 μl PBS and transferred to a clean tube to avoid contamination of the elute with proteins bound to the tube wall. The tube was placed on the magnet, the supernatant was removed and 20 μl of premixed NuPage LDS Sample Buffer and NuPAGE Sample Reducing Agent was added and mixed gently to resuspend the beads. The tubes were then incubated at 70 °C for 10 min. Finally, the tube was placed on the magnetic holder to pull down the beads and the supernatant was loaded onto the gel. A negative control in the form of an IgG control IP, where the equivalent concentration of rabbit IgG as the antibody concentration was incubated with the Dynabeads, was also performed to ensures the specificity of the results. Additionally, a positive control was employed, loading the total lysate to ensure the IP band is at the right molecular weight.

4.13. NGAL ELISA

Buffers were prepared according to the manufacturers' instructions. Plates were coated with 50 µl/well of HRP-conjugated NGAL antibody. 50 µl of calibrators, internal controls and diluted samples (1:200) were added to the wells and incubated for 30 min at room temperature on an orbital shaker. The wells were washed three times with 300 µl/well wash buffer. Excess liquid was removed from plates by tapping gently on absorbent paper. 100 µl TMB solution was dispensed to each well and incubated for 15 min at room temperature, covered from light. 100 µl/well stop solution was added to the wells and mixed gently by pipetting. Results were read at 450 nm on a plate reader.

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Transparency document

The Transparency document associated this article can be found, in online version.

Declaration of competing interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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