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The role of DNA damage as a therapeutic target in autosomal dominant polycystic kidney disease

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic kidney disease and is caused by heterozygous germ-line mutations in either *PKD1* (85%) or *PKD2* (15%). It is characterised by the formation of numerous fluid-filled renal cysts and leads to adult-onset kidney failure in ~50% of patients by 60 years. Kidney cysts in ADPKD are focal and sporadic, arising from the clonal proliferation of collecting-duct principal cells, but in only 1–2% of nephrons for reasons that are not clear. Previous studies have demonstrated that further postnatal reductions in *PKD1* (or *PKD2*) dose are required for kidney cyst formation, but the exact triggering factors are not clear. A growing body of evidence suggests that DNA damage, and activation of the DNA damage response pathway, are altered in ciliopathies. The aims of this review are to: (i) analyse the evidence linking DNA damage and renal cyst formation in ADPKD; (ii) evaluate the advantages and disadvantages of biomarkers to assess DNA damage in ADPKD and finally, (iii) evaluate the potential effects of current clinical treatments on modifying DNA damage in ADPKD. These studies will address the significance of DNA damage and may lead to a new therapeutic approach in ADPKD.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a common monogenic condition, with an estimated population prevalence of 1 in 1000 (Refs 1, 2). It is caused by loss-of-function heterozygous germ-line mutations in *PKD1* (16p13.3; OMIM 601313; 85% of cases), *PKD2* (4q21; OMIM 173910; 15% of cases) or rarely other genes (such as *GANAB* and *DNAJB11*) (Refs 3–5). *PKD1* and *PKD2* encode, polycystin-1 and -2, respectively, which are transmembrane proteins that localise and regulate primary ciliary function, such that ADPKD is classified as a renal 'ciliopathy' (Refs 3, 6). ADPKD is characterised by the bilateral, progressive formation and growth of numerous microscopic, fluid-filled kidney cysts and associated with the deregulation of multiple intracellular signalling pathways (Refs 3, 7). Kidney cyst formation has been hypothesised to begin *in utero* (or during early life) because of reduced intracellular levels of polycystins (Refs 8, 9). Throughout life, kidney cysts continually grow by ~5% per year because of cystic epithelial cell proliferation and fluid secretion, resulting in the gradual loss of healthy kidney tissue (Ref. 3). The severity of renal dysfunction is determined by the number of kidney cysts that develop during life (Ref. 10), and ~50% of patients develop end-stage kidney disease (ESKD) before the age of 60 (Refs 1, 3). Other disease complications are variable and include hypertension, polycystic liver disease and intracranial aneurysms (Ref. 3).

Despite significant progress in understanding the pathogenesis of ADPKD, the mechanisms that 'trigger' kidney cyst formation during life remain unclear, and there are no disease-modifying interventions that specifically target the prevention of cystogenesis (Ref. 3). Several studies have suggested a link between DNA damage, oxidative stress and kidney cyst formation, and therefore the aims of this review were to: (i) critically analyse these data and determine their applicability to ADPKD; (ii) evaluate methods that can be used to assess DNA damage in PKD and (iii) determine the potential for current therapeutic approaches to alter DNA damage in ADPKD.

DNA damage and the DNA damage response (DDR) in normal health and in chronic kidney disease

In normal health, the DNA in each of the 10^{13} cells that make up the human body encounters tens of thousands of potentially damaging agents and processes per day, which can result in loss of fidelity in the DNA code (Ref. 11). This injury can stall DNA replication and

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transcription, and if not repaired correctly, can lead to permanent mutations that threaten genome integrity and cause disease (Refs 12, 13). DNA damage is categorised into two types: (1) endogenous DNA damage, which is caused by metabolic processes within the cell itself, such as spontaneous reactions (e.g. hydrolysis) or reactive oxygen and nitrogen species (ROS and RNS); and (2) exogenous DNA damage, which is potentially preventable and occurs when cells are exposed to physical damage or chemical agents such as ultraviolet (UV) radiation (Refs 12, 14). Oxidative stress, which refers to a state of imbalance where the production of ROS exceeds the regulatory capacity of antioxidants, is an important cause of endogenous DNA damage (Ref. 15). Excess ROS react with different components of DNA and causes a variety of DNA lesions including base modification, inter- and intra-strand crosslinks and DNA breaks (Ref. 15). Together, spontaneous DNA damage from endogenous sources can cause up to 10^5 lesions per cell per day (Refs 12, 16). The relative contributions by endogenous and exogenous DNA damage to disease is unknown, however, it has been suggested that exogenous factors are causal in 75–80% of cancer cases (Refs 17, 18).

To defend against oxidative stress, genes that encode antioxidant enzymes, transcription factors and proteins are activated or silenced in an attempt to maintain redox balance (Ref. 19). For example, glutathione (a key antioxidant) will donate its electron to H_2O_2 to form water and oxygen, reducing the incidence of free radicals (Ref. 19). If antioxidant defence is unsuccessful, undesirable DNA modifications may occur (Ref. 19). DDR is a series of cellular signalling processes and enzymatic activities that is initiated by DNA damage (Ref. 20). It is facilitated by ∼450 proteins that function to: (1) identify the site of DNA damage, (2) recruit DNA repair factors to the site of damage and (3) repair the physical DNA lesion (Refs 20, 21). In the presence of DNA damage, the histone variant, H2AX, is phosphorylated on the 139th serine residue to form γ-H2AX, and this acts as a signal to facilitate DNA repair (Ref. 14). The DDR is primarily mediated by Ataxia Telangiectasia and Rad3-related kinase (ATR), Ataxia-Telangiectasia Mutated (ATM) and DNA-dependent protein kinase (DNA-PK), which trigger alternative DNA repair pathways, dependent on the type of DNA lesion that has occurred (Ref. 20). As part of the DDR, ATR and ATM phosphorylate checkpoint kinase 1 (Chk1) and 2 (Chk2), respectively, which maintains cells in an inactive state and prevents entry into mitosis to allow repair to occur (Ref. 20).

ADPKD also shares some common histological features with other forms of chronic kidney disease (CKD), such as tubulointerstitial fibrosis and inflammation (Ref. 6). Published data over the last 30 years have described an association between DNA damage and progression of CKD (Refs 22–31). Epidemiological data indicate that poor kidney function is correlated with an increased risk of cancer, and this has been attributed to genomic instability, increased cellular DNA damage and impaired DNA repair (Refs 22, 23). Cengiz *et al.* were the first to demonstrate that uraemia was associated with abnormalities in chromosome structure and an increased rate of sister chromatid exchange (an indicator of carcinogenic or mutagenic potential) in lymphocytes (Ref. 24). Subsequent studies have also shown that increasing genetic damage is associated with decline in kidney function (Refs 25, 26). Furthermore, in CKD, DNA repair is inhibited (Refs 27, 28), and higher rates of oxidative stress are reported (Refs 29, 30). In this regard, elevated serum 8-hydroxy-2′-deoxyguanosine (8-OHdG; a biomarker of oxidative stress) also correlated the progression of kidney function decline in CKD patients (Ref. 31). These data suggest that DNA damage may have a pathogenic role in mediating the progression of CKD.

Genetic and cellular mechanisms underlying focal kidney cyst formation in ADPKD

One of the long-standing conundrums of ADPKD is that kidney cyst formation is focal and arises from only 1–2% of nephrons even though all cells from an affected individual carry one copy of the mutated PKD gene (Refs 8, 32). Previous studies suggest that further postnatal reductions of *PKD1* (or *PKD2*) precedes, and is necessary, for initiation of kidney cysts (Refs 33–36). The gene dose-dependent model of cystogenesis in ADPKD was described by Rossetti *et al.*, who demonstrated that incompletely penetrant *PKD1* alleles were associated with mild disease severity in humans (Ref. 35). Subsequently, Hopp *et al.* demonstrated that the hypomorphic *PKD1* p.R3277C (RC) allele is also associated with a milder ADPKD phenotype, where $Pkd1^{+/null}$ mice are do not develop kidney cysts, $Pkd1^{RC/null}$ mice exhibit rapidly progressive disease, and $Pkd1^{RC/RC}$ animals develop kidney cysts gradually (Ref. 36). It is believed the healthy *PKD* gene allele inherited from the parent without ADPKD provides sufficient levels of polycystin protein, but when this allele is inactivated within an individual renal tubular cell by a somatic 'second hit' (mutation or by stochastic mechanisms, as discussed further below), a kidney cyst develops (Ref. 37). As the cyst enlarges, it separates itself from the tubule and becomes an isolated, self-contained structure (Ref. 38). The kidney cyst then undergoes aberrant proliferation, increasing in cell number and size and enlarges by fluid secretion into the cyst lumen (Ref. 38). Over time, thousands of cysts burden the kidney, varying in diameter from one to several centimetres and the kidney can weigh up to 5 kg (Ref. 6).

Thus, the 'two-hit' model for cyst formation suggests that loss of heterozygosity because of a somatic or 'second-hit' mutation in the healthy allele causes cyst formation (Ref. 39). This hypothesis is supported by extensive *in vitro* and *in vivo* studies (Refs 40–47), and is described in Figure 1a. Evidence of loss of heterozygosity was initially demonstrated by genetic analysis of cystic epithelium, which revealed loss of the wild-type copy of *PKD1* (Refs 40, 41). Moreover, somatic inactivation of *PKD2* by homologous recombination in adult mice was causative in renal and hepatic cyst formation (Ref. 42). Most recently in support of this hypothesis, it was found that in a cohort of nine ADPKD patients, somatic mutations of *PKD1* or *PKD2* were present in all and occurred in 90% of kidney cysts (Ref. 47).

Other hypotheses to explain the reduction in *PKD* dosage include an age-related decline (Ref. 35), random fluctuations during development and/or influence of environmental factors (Ref. 6). Regarding the latter, a 'third hit' hypothesis, namely proliferative signals, also appears to be essential for kidney cyst formation (Refs 46, 48). This 'third hit' was proposed to explain the discrepancy between the slower onset of focal cystic disease when *PKD1* was inactivated in adult rather than infantile mice (Ref. 46), and the rapid development of kidney cysts in response to a third-hit, such as renal ischaemia-reperfusion injury (Ref. 48).

Finally, and more recently, the 'snowball effect' of cyst formation has also been described. Leonhard *et al.* observed that cysts formed in clusters, and PKD-related signalling (pSTAT3, pCREB, pAkt, pERK 1/2 and LCN2) was upregulated in tissue surrounding these clusters, suggesting that paracrine influences from a founder cyst may trigger the formation of new cysts (Ref. 49). In addition, recent *in vitro* data indicate cyst fluid itself may contain accelerating paracrine trophic factors to induce regional cystic kidney disease (Ref. 50).

Despite some common elements in these hypotheses, the unifying molecular pathways that link them and explain the focal nature of cyst formation in ADPKD has not been clarified. As

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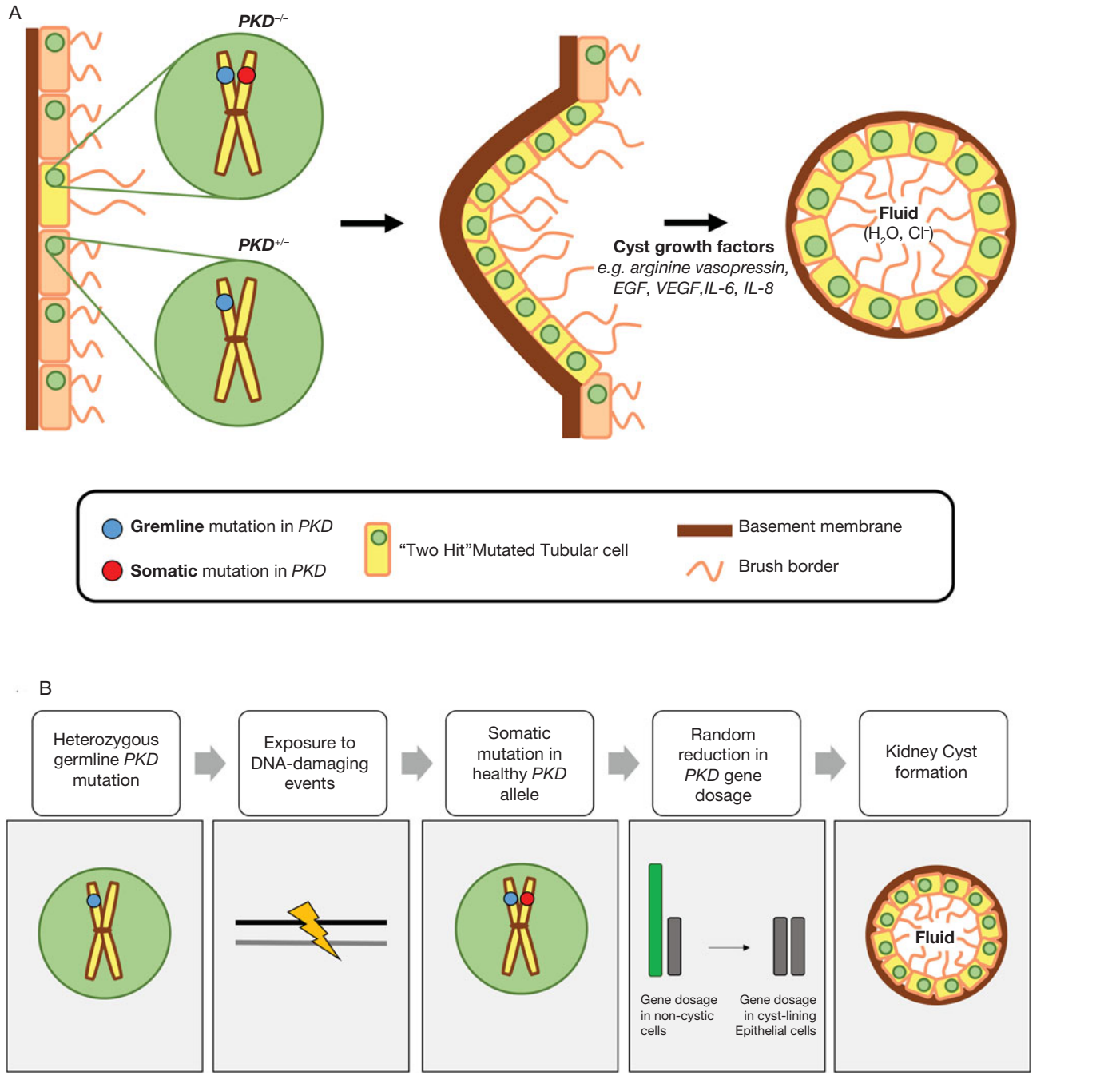

Fig. 1. (a) *The 'two-hit' model of cystogenesis in autosomal dominant polycystic kidney disease (ADPKD)*. In ADPKD, all tubular epithelial cells exhibit a heterozygous germline mutation in either *PKD1* or *PKD2* (shown by the blue circle), and this is the 'first-hit'. A loss of heterozygosity by an acquired or somatic mutation of the normal *PKD* allele in an individual cell is required to initiate cyst formation (shown by the red circle), and this is the 'second-hit'. This epithelial cell then undergoes proliferation, potentiated by cyst growth factors such as arginine vasopressin epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and interleukin-8 (IL-8). The cyst eventually detaches from the original nephron and progressively increases in size because of epithelial cell proliferation, dedifferentiation and abnormal fluid secretion. (b) *A hypothetical model to explain the role of DNA damage in autosomal dominant polycystic kidney disease (ADPKD)*. Exposure to DNA-damaging events is causal in the somatic mutation of the healthy *PKD* allele, resulting in a random reduction in gene dosage and resulting in kidney cyst formation. These DNA-damaging events may be because of endogenous factors (ageing, replication stress from *PKD1* duplication, increased oxidative stress, epigenetic modifications and inflammation) and/or exogenous sources (elevated NaCl concentration in the inner medulla).

the severity of ADPKD is directly related to the occurrence frequency of this focal cyst-initiating event, targeting this mechanism is likely to be the key to preventing ESKD.

Potential role of DNA damage in reducing PKD gene dose in ADPKD

Previous studies have shown that alterations in DNA repair proteins are associated with development of human ciliopathies (Refs 51–55). For example, Chaki *et al.* found that genes encoding DNA repair proteins (*MRE11*, *ZNF423* and *CEP164*) are mutated in nephronophthisis-related ciliopathies (Ref. 54). Moreover, *CEP164* deficiency was associated with systemic DNA damage, increased sensitivity to DNA damaging events, and impaired DNA damage signalling (Refs 54, 55). *MRE11* and *ZNF423* both play a role in the ATM-Chk2 DDR pathway, recruiting ATM to sites of damage, and *CEP164* is activated by ATM and ATR, as well as necessary for Chk1 activation. Further experimental and clinical evidence linking DNA damage and cystic kidney disorders, can be summarised as follows:

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Preclinical studies

Kidney cysts in ADPKD and ARPKD originate mainly from tubular epithelial cells present in nephrons from the inner medulla (Ref. 56). Dmitrieva *et al.* demonstrated that murine inner medullary tubular epithelial cells are more susceptible to DNA damage compared with those from the renal cortex (Ref. 57). Furthermore, DNA repair in inner medullary cells was impaired in response to total body irradiation, as evidenced by the reduced H2AX phosphorylation and lack of newly synthesised DNA (Ref. 57). Therefore, it is possible that the susceptibility of the distal nephron to renal cyst formation could be related, in part, to the more hostile environment causing DNA damage of inner medullary tubules, together with impaired DNA repair.

Nek8 is a mitotic kinase that regulates intracellular levels of polycystins and is mutated in the *jck* mouse model of PKD and human nephronophthisis (a cystic kidney disease) (Refs 58, 59). Choi *et al.* reported that knockdown of Nek8 *in vitro* causes the accumulation of double-strand breaks (DSBs) in DNA, irregular origin firing (unscheduled DNA replication), and a higher likelihood of replication fork collapse (Ref. 60). *In vivo*, kidney DNA damage, measured by γ-H2AX, was increased in *jck* mice compared with the wild-type group (Ref. 60). Activation of DNA repair signalling has also been associated with mutation in *Sdccag8* (gene mutation causing nephronophthisis), characterised by increased γ-H2AX levels and ATM activity both *in vitro* and *in vivo* (Ref. 61). Finally, kidney tubular cells extracted from 8-week-old *Pkd1* knockout mice revealed an increase in DNA breaks, as determined by the alkaline comet assay (Ref. 62). However, analysis of γ-H2AX by western blotting in kidney lysates of these mice showed no differences when compared with wild-type (Ref. 62).

Human studies

Evidence that DNA damage is increased in human ADPKD is lacking. Interestingly, Cengiz *et al.* reported the highest levels of sister chromatid exchange in patients with PKD (n = 3) compared with other causes of uraemia, providing preliminary clinical evidence for a link between DNA damage and ADPKD (Ref. 24). Ta *et al.* showed that DNA damage, measured by γ-H2AX positive immunostaining, is increased in human ADPKD kidney cyst-lining epithelial cells (Ref. 63). Thus, further investigation is required to determine whether DNA damage has a direct role in human ADPKD.

Potential reasons for increased DNA damage in ADPKD

Evidence from preclinical and clinical studies indicate that DNA damage is increased in renal ciliopathies and renal cystic diseases. Most of these studies attribute this increase to defective DDR, resulting in impaired DNA repair and DNA damage accumulation. For example, Choi *et al.* hypothesised that Nek8 may play an integral role in preventing the accumulation of DNA damage through its interaction with the DNA repair proteins, ATR and Chk1 (Ref. 60). However, the mechanisms for increased DNA damage in PKD are not clear and could include several other possibilities.

PKD1 *gene is prone to replication stress and DNA damage because of large mirror repeat sequences*

Replication stress is a term that describes barriers to DNA replication, including variations in DNA, complex structures that are difficult to replicate and/or exhaustion of the nucleotide pool (Ref. 64). *PKD1* contains a polypurine-polypyrimidine (Pu-Py) tract made up of large mirror repeat sequences that form complex, 'difficult to replicate' structures (Ref. 65). Liu *et al.* found evidence that these non-B secondary DNA structures from the *PKD1* gene lead to replication fork stalling, followed by DSBs and subsequent activation of DNA repair signalling (Ref. 65). Thus, the observed DNA damage may, in part, be because of replication stress associated with *PKD1* replication. Further investigation is required to examine whether this could be the cause of the 'second hit' mutation to the healthy allele in ADPKD. Lea *et al.* reported that the presence of the long polypyrimidine tract led to aberrant splicing in human *PKD1*, resulting in 61.5% of *PKD1* transcripts having premature stop codons (Ref. 66). This, in turn, resulted in decreased levels of full-length mRNAs, and formation of a lower molecular weight, truncated form of polycystin-1 (Ref. 66). Lea *et al.* hypothesised that the decreased full-length polycystin-1 from normal alleles causes reduced polycystin signalling below 'threshold' levels, resulting in cyst initiation and development (Ref. 66).

Genetic mutations associated with cystic kidney diseases increase susceptibility to DNA damage

Cells isolated from ADPKD patients are more susceptible to DNA damage after irradiation compared with healthy controls (Ref. 67). Battini *et al.* provided *in vivo* evidence that conditional knock-out (KO) of *PKD1* was associated with significant centrosome amplification, and the loss of polycystin-1 resulted in mitotic catastrophe and genomic instability during disease progression in this model (Ref. 68). Both Li *et al.* and Battini *et al.* proposed that the 'second hit' or somatic mutation to the *PKD* gene is required for dysregulated polycystin-1 expression and provides an explanation for increased genomic instability (Refs 67, 68). The increased genomic instability may also explain the phenotypic variability in ADPKD and sporadic cyst formation in few nephrons (Ref. 68). In addition, a cross-species meta-analysis of conserved biological pathways in ADPKD also suggested that transcriptomic alterations related to genomic instability were associated with regulating cyst formation (Ref. 7). Finally, a cohort study from Taiwan suggested that patients with ADPKD have higher risk of developing liver, colon and renal cancer compared with those without the disease (Ref. 69). However, this has not been confirmed by all studies, and overall, the relationship between ADPKD and cancer incidence remains conflicting (Refs 70, 71). For example, in contrast, Wetmore *et al.* reported that renal transplant recipients with PKD were 16% less likely to develop cancer compared with non-PKD patients (Ref. 72).

Increased oxidative stress results in subsequent endogenous DNA damage

Kidney cystic disorders are characterised by an increase in oxidative stress, which may contribute to endogenous DNA damage (Refs 62, 73–75). A case-control study showed that ADPKD patients had higher levels of aminothiols (marker of oxidative stress) compared with healthy controls (Ref. 74). The redox imbalance in CKD may be because of reduced estimated glomerular filtration rate (eGFR) and/or hypertension (Refs 73, 74). In particular, elevated levels of plasma total homocysteine (a component of the antioxidant defence system) are associated with reduced eGFR, and may provide one explanation for increased oxidative stress in ADPKD (Ref. 73). More direct evidence was provided by Cassini *et al.*, who demonstrated that 8-OHdG was expressed in cyst-lining cells of *Pkd1* mutant mice (Ref. 62). Finally, Nowak *et al.* demonstrated that acute intravenous infusion with ascorbic acid (an inhibitor of free radicals) improved brachial artery flow-mediated dilation (a measure of endothelial dysfunction) in

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humans with ADPKD, but had no effect on controls, indicating the presence of vascular endothelial oxidative stress (Ref. 75).

Epigenetic changes results in DNA damage

A limited number of studies have examined the functional significance of epigenetics in ADPKD (Refs 76–78). Woo *et al.* observed that *PKD1* was hypermethylated, and inhibiting DNA methylation ameliorated cyst formation *in vitro* (Ref. 76). Moreover, the non-coding RNA, miR-182-5p, was found to be novel regulator of progression in conditional *PKD1/2*-deficient mice (Ref. 78). Genes that have undergone epigenetic modifications, such as methylation of the DNA base, cytosine, may be more prone to oxidative stress later in life, as well as exhibiting reduced ability to repair oxidised DNA bases (Ref. 79). In the context of Alzheimer's disease, hypomethylation of disease-related genes led to overproduction of ROS-producing proteins, and hypermethylation led to diminished capacity to repair 8-OHdG (a product of oxidative stress) (Ref. 79). Therefore, this observed DNA damage may be a consequence of epigenetic modifications to the *PKD* gene, as hypermethylation may result in increased oxidative stress, reduced base excision repair capacity and subsequent DNA lesions.

Increased renal interstitial inflammation causes DNA damage

There is clear interplay between inflammation, DNA damage and DNA repair (Ref. 80), and inflammation is also a key pathological feature of PKD, as reviewed previously (Ref. 81). In both acute and chronic inflammation in the kidney, neutrophils, eosinophils and macrophages are activated and as a defence mechanism, large volumes of ROS and RNS are produced, which may have genotoxic effects as well as inhibiting the DDR response (Ref. 80). Cassini *et al.* investigated this hypothesis and found reduced DNA breaks in $PKD1^{-/-}/MCP1^{-/-}$ mice compared with $PKD1^{-/-}/MCP1^{+/+}$ mice (Ref. 62). Thus, observed DNA damage may be a result of interstitial pro-inflammatory signalling around kidney cysts.

Exposure to endogenous and exogenous factors results in DNA damage

Both endogenous and exogenous DNA-damaging events may also contribute to DNA damage observed in both CKD and cystic kidney disease. Ageing is associated with genome instability because of endogenous factors, as DNA undergoes time-dependent deterioration from spontaneous biological reactions (Ref. 12). Moreover, previous *in vitro* studies have demonstrated that acute increases in NaCl inhibits the DDR (evidenced by lack of H2AX and Chk1 phosphorylation), resulting in the accumulation of DNA breaks and impaired DNA repair (Ref. 82). In addition, both *in vitro* and *in vivo* studies have demonstrated that angiotensin II can also trigger DNA damage (Refs 83–85). The increase in damage could be attenuated by administration of an angiotensin II type-1 receptor blocker (candesartan) and antioxidants (*N*-acetylcysteine and α-tocopherol), highlighting that angiotensin II causes DNA damage via this receptor and subsequent formation of oxidative stress (Refs 83, 85).

Exogenous events such as renal ischaemia-reperfusion stimulate phosphorylation of DDR proteins, ATM, H2AX, Chk2 and p53 *in vivo*, which is likely because of increased ROS (Ref. 86). As mentioned earlier, ischaemia and nephrotoxic injury is associated with exacerbated cyst burden in PKD (Refs 48, 87, 88). Therefore, DNA lesions resulting from oxidative damage because of ageing, high sodium content of the inner renal medulla, high intra-renal levels of angiotensin II, ischaemia or nephrotoxic injury are other potential explanations for increased DNA damage in ADPKD. The role of chronic exposure to endogenous and exogenous DNA-damaging events in disease pathogenesis is important to consider because of the incidence of somatic mutations in ADPKD pathogenesis. Alexandrov *et al.* demonstrated that 'clock-like' mutational signatures present in clear cell and papillary renal cancers originate from continuous exposure and reabsorption of mutagens in the proximal tubular epithelium of the kidney (Ref. 89), highlighting a causal role for endogenous and exogenous factors in the development of mutations and disease.

It is unlikely that any of these possible explanations occur in isolation in the complex microenvironment of the polycystic kidney. Therefore, a combination of replication stress associated with the *PKD* gene, oxidative stress, exposure to endogenous and exogenous factors, inflammation, genetic mutations and epigenetic changes, most likely explains the observed increase in DNA damage in ADPKD.

Biomarkers that could be used to assess DNA damage in ADPKD

Multiple techniques can be used for the detection, analysis and quantification of DNA damage, in both preclinical and clinical settings. These techniques vary in specificity, sensitivity and ease of administration, and each have their own advantages and disadvantages. The key methods for measuring DNA damage in the current literature are: (i) the comet assay, (ii) γ-H2AX and (iii) 8-OHdG, and these are summarised in Table 1 and below.

Preclinical biomarkers

Comet assay: Overall, the comet assay is widely used and provides a reliable assessment of DNA damage levels. The benefit of the comet assay is that it can be performed in cells isolated non-invasively from humans (e.g. peripheral blood lymphocytes), however it is unclear whether DNA damage detected in these cells is an accurate reflection of damage in tissues of interest (Ref. 90). Previous studies have used the comet assay to examine DNA damage in healthy individuals compared with those on dialysis (Ref. 91), and these demonstrated that DNA damage was increased in the latter when performed on peripheral blood lymphocyte cells (Ref. 91). Choi *et al.* also observed that an increased tail moment, representative of increased DNA damage, when neutral comet assays were performed on HeLa cells with Nek8 knockdown, indicating increased DSB (Ref. 60).

γ-H2AX: The measurement of γ-H2AX is also limited to a preclinical setting, using *in vitro* and *in vivo* models of disease, as it requires tissue and cannot be measured from routine specimens collected from human patients such as blood and urine. The advantage of γ-H2AX is that it provides insight into potential molecular pathways involved in the body's response to DNA damage, and its upstream effectors or downstream targets may be related to disease causality. Overall, immunoblotting is less sensitive and is not informative of the location or nature of γ-H2AX foci (Ref. 92). This technique would be useful in the examination of ADPKD tissue as the localisation of γ-H2AX in cyst-lining cells compared with non-cyst-lining cells would be of interest. However, the quantification of immunostaining can be challenging as there is variable background level of γ-H2AX associated with DNA replication occurring in S-phase cells (Ref. 93). To overcome these difficulties, detection of other proteins involved in DNA repair could act as a surrogate measure of DNA damage (Ref. 93). However, immunostaining for these other proteins (e.g. p53, ATR and ATM) is not equivalent to γ-H2AX, as most DNA repair proteins already exist in the nucleus. Therefore, their rates of accumulation may not

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Table 1. A summary of methods that could be used to assess DNA damage in cystic kidney diseases

Measure	Description	Method	Refs
Comet assay	Cells are embedded in a thin layer of agarose gel, then lysed using a detergent and high salt to remove cellular proteins and immobilise DNA. Following electrophoresis and staining with a DNA-binding dye, the damaged DNA fragments migrate from the nucleus to form a 'comet' structure, including a head and tail. The 'head' is made up of intact or undamaged DNA, whereas the 'tail' consists of damaged DNA (single- or double-stranded; SSBs and DSBs). The intensity of staining in the comet tail relative to the head is a direct measure of the number of DNA breaks.	• Single-cell gel electrophoresis • Common variants include alkaline and neutral single-cell gel electrophoresis, and the use of lesion-specific enzymes. • The most commonly used specimen is peripheral blood lymphocytes, with a handful of studies using whole blood, and cells from salivary glands.	90, 91
γ-H2AX	H2AX is a variant of H2A, one of the five families of histones that package and organise eukaryotic DNA into chromatin. Within minutes of DSB formation, large numbers of phosphorylated H2AX, or γ-H2AX, form in the chromatin surrounding the site of damage. DNA damage repair kinases, ATR, ATM, DNA-PK and the MRN complex are responsible for the phosphorylation of H2AX. γ-H2AX acts as a signal to assist DNA damage repair by: (1) increasing DNA accessibility to allow the recruitment and accumulation of specific repair proteins at DNA ends, (2) facilitating DSB repair by anchoring the DNA ends and (3) modulating cell cycle checkpoints to give DNA additional time for repair. After DNA is repaired, γ-H2AX is dephosphorylated. The kinetics of γ-H2AX dephosphorylation appear to follow two distinct phases: (1) a fast phase that involves rapid DSB repair and dephosphorylation within minutes to hours, and (2) a slower phase, where DSB repair may take several days to months, resulting in persistently higher baseline γ-H2AX.	• Immunostaining: counting γ-H2AX-containing structures in images of cells and tissues. • Immunoblotting: measuring overall γ-H2AX protein levels.	14, 92–96
8-Hydroxy-2′-deoxyguanosine (8-OHdG)	The hydroxyl radical ($HO^{\cdot}$) interacts with the nucleobases of the DNA strand, such as guanine, to form 8-OHdG. 8-OHdG has been established as an important biomarker of oxidative stress. 8-OHdG does not persist in DNA because of its mutagenic properties (e.g. G:C→A:T transversions). Its removal by DNA repair systems including base excision repair, nucleotide excision repair, mismatch repair and prevention of incorporation, results in the excretion of 8-OHdG in urine. The amount of urinary 8-OHdG is thought to be representative of whole-body oxidative DNA damage.	• Immunohistochemical methods for example ELISA • Single-cell gel electrophoresis • High-pressure liquid chromatography, with mass spectrometric or electrochemical detection	97–99

ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; DNA-PK, DNA-dependent protein kinase; DSB, double-stranded break; ELISA, enzyme-linked immunosorbent assay; MRN, MRE11-RAD50-NBS1; SSB, single-stranded break; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; γ-H2AX, gamma-H2AX.

correspond directly to onset of DNA damage, as background protein levels may be present (Ref. 93). Alternatively, if the protein of interest is also phosphorylated *de novo* (in a similar nature to γ-H2AX), using an antibody to its phosphorylated form may yield more accurate results (Ref. 93).

Clinical biomarkers

8-Hydroxy-2′-deoxyguanosine (8-OHdG): The measurement of 8-OHdG could be easily incorporated into clinical research studies as it can be measured from routinely collected specimens, and the availability of enzyme-linked immunosorbent assay (ELISA) provides a simple, high-throughput technique. However, 8-OHdG lacks sensitivity, as it is an indicator of DNA damage caused by oxidative stress exclusively, excluding other types of DNA damage. Furthermore, the antibodies available for 8-OHdG measurement have been previously criticised for the lack of specificity and tendency for overestimation, highlighting the importance of controls and careful data interpretation (Ref. 100). Overall, the measurement of 8-OHdG would primarily demonstrate the contribution of endogenous and exogenous factors to DNA damage in ADPKD. Similar to the comet assay, 8-OHdG levels have also been compared in healthy and dialysed individuals, and a review of all studies revealed higher oxidative stress in individuals undergoing haemodialysis (Ref. 91).

In summary, there is no perfect method for assessing DNA damage in either experimental or clinical context. The comet assay is regarded as the 'gold-standard' for assessment of DNA damage (Ref. 101). In preclinical studies, the comet assay provides an indication of the presence or absence of DNA damage, encompassing all the different types of DNA damage, and can be quantified using specific software. Despite this, γ-H2AX tends to be routinely used in preclinical studies (instead of the comet assay), probably because it can be measured using routine laboratory techniques such as immunoblotting and immunohistochemistry. In contrast, in clinical research studies, urinary or serum 8-OHdG, is a practical method to use as it can quantified using ELISA. Therefore, based on our analysis of the methodological data, our recommendation is for investigators to use a combination of methods to verify the hypothesis.

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Clinical implications and applications

It is possible that current therapies for ADPKD could, in part, be protective because of their effect on DNA damage and further studies might be helpful in addressing this hypothesis. In this regard, current therapies in clinical practice, as well as those under investigation, and their potential link to DNA damage are summarised below and in Table 2.

Angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs)

Previous *in vitro* and *in vivo* studies have demonstrated that angiotensin II can trigger DNA damage. Schmid *et al.* found that increased levels of angiotensin II caused a significant increase in DNA strand breaks (measured by the comet assay) in isolated perfused mouse kidneys (Ref. 84). In addition, γ-H2AX and 8-OHdG were detected in angiotensin II-treated cells (Ref. 84). Treatment of pig kidney cells with angiotensin-II led to an increase of DNA damage (up to 15-fold measured by the comet assay) and enhanced formation of ROS (Ref. 83). This increased damage could be attenuated by administration of an ARB (candesartan) and antioxidants (*N*-acetylcysteine and α-tocopherol) (Ref. 83). These findings were consistent *in vivo*, where treatment with angiotensin II led to increased oxidative stress (measured by ROS formation), elevated DSBs in the kidney (measured by γ-H2AX) and increased poly (ADP-ribose) polymerase activity (PARP; a DNA repair protein) (Ref. 103). All these effects were reversed with administration of candesartan (Ref. 103). Therefore, the small benefit of lower blood pressure targets in reducing cyst growth in ADPKD mediated by ACE inhibitors and ARBs (Ref. 102), may be related to reduced angiotensin levels and DNA damage inhibition.

Arginine vasopressin (AVP) signalling

The functional role of AVP in renal cyst progression has been confirmed in human ADPKD, where administration of a V_2 receptor antagonist (tolvaptan) resulted in modest reductions in total kidney volume increase and eGFR decline (Refs 104–106). There is limited evidence to suggest that AVP levels directly influence DNA damage or DNA repair. In male Wistar rats, vasopressin infusion exhibited antioxidant properties, reducing oxidative stress and demonstrating a cardioprotective effect against ischaemia-reperfusion injury (Ref. 107). Furthermore, increased cAMP levels, which AVP is known to upregulate, have demonstrated a protective effect against the DNA damaging agents (cisplatin and etoposide) in an *in vitro* model of chronic myeloid leukaemia (Ref. 108). However, dehydration and elevated vasopressin levels have been associated with increased oxidative stress, contributing to neurovascular and cognitive defects in adult mice (Ref. 109). It is possible that DNA damage is involved in the earlier stages of disease as a trigger for initial kidney cyst formation, whereas AVP acts as the driver for continued proliferation.

Modifiable dietary factors

Modifiable dietary factors, namely dietary sodium intake (Refs 130–132) and caloric restriction (Refs 133–135), may mediate the progression of ADPKD. *In vitro*, acute increases in NaCl inhibits the DDR, resulting in the accumulation of DNA breaks and impaired DNA repair (Ref. 82). Of note, mouse inner medullary collecting duct cells were able to adapt when exposed to a high NaCl environment, with continued rapid proliferation and no evidence of apoptosis (Ref. 57). However, the cells did not activate the DNA repair signalling, as evidenced by the lack of H2AX phosphorylation, and had persistent numerous DNA breaks (Ref. 57). When NaCl level was decreased, DNA repair signalling was reactivated, indicated by H2AX phosphorylation (Ref. 57). Therefore, a clear relationship exists between higher salt, increased DNA damage and impaired DNA repair *in vitro*, but these data need further verification *in vivo*.

Recent studies have also suggested a protective role for food restriction in the context of ageing and DNA damage, and this has been reviewed previously (Refs 136–138). Vermeij *et al.* observed that in mice deficient in the DNA excision repair gene ($Ercc^{\Delta/-}$), caloric restriction of 30% increased median and maximal lifespans of by three-fold (delaying accelerated ageing), and reduced the number of γ-H2AX foci (Ref. 139). Therefore, the beneficial effect of food restriction in murine ADPKD may be mediated, at least in part, by reductions in DNA damage.

Nicotinamide

Nicotinamide is the active form of vitamin B_3 or niacin, and is a precursor for the synthesis of nicotinamide adenine dinucleotide (Ref. 122). Zhou *et al.* discovered that administration of nicotinamide drastically reduced cyst formation in $Pkd1^{-/-}$ embryos and *Pkd1* KO mice (Ref. 121). However, the mechanism of action proposed for the effect of nicotinamide on delaying cyst growth was via sirtuin 1 inhibition, rather than DNA damage inhibition (Ref. 121). Furthermore, Zhou *et al.* found that *Pkd1* and *Sirt1* double KO mice had no further delay in cyst growth compared with just *Pkd1* KO mice, suggesting that nicotinamide reduces cyst growth by specifically inhibiting sirtuin 1, and is unlikely to act via other pathways (Ref. 121). Currently, a pilot randomised controlled trial (NIAC-PKD2; ClinicalTrials.gov Identifier: NCT02558595; https://clinicaltrials.gov/ct2/show/NCT02558595) has been completed at the University of Kansas Medical Center to observe the effects of niacinamide (reduced form of niacin) on kidney cyst growth and markers of kidney disease progression. Nicotinamide promotes DNA repair by providing a substrate for PARP-1 activity, an energy reserve for ATP-dependent DNA repair, and preserving the integrity of PARP-1 (Ref. 122). Moreover, the ONTRAC study found that 500 mg of nicotinamide twice daily was associated with a lower rate of new non-melanoma skin cancers in a high-risk population (Ref. 123).

Other investigational treatments

Metformin: Recent studies, focused on drug repurposing, have revealed significant therapeutic potential for metformin in the treatment of ADPKD (Refs 110, 111). A phase II, double-blinded, randomised placebo-controlled trial (TAME; ClinicalTrials.gov Identifier: NCT02656017; https://clinicaltrials.gov/ct2/show/NCT02656017) is underway to examine efficacy, feasibility, safety and tolerability of metformin use in patients with ADPKD (Ref. 140). In addition, a clinical trial, called Implementation of Metformin therapy to ease decline of kidney function in PKD (IMPEDE-PKD), by the Australasian Kidney Trials Network (AKTN) is in development (https://aktn.org.au/trials/trials-in-development/impede-pkd/). The relationship between metformin and DNA damage remains unclear, where experimental evidence suggests both a harmful and protective role (Refs 112–119). Epidemiological evidence suggests type-2 diabetic patients on metformin have lower cancer incidence (Ref. 114), and there is *in vivo* and *in vitro* evidence that suggests metformin reduces tumour growth rates (Refs 115–118). Moreover, metformin reduced insulin-induced DNA damage and oxidative stress in the Zucker diabetic fatty rat (Ref. 119). However, some studies have reported elevated oxidative stress and increased DNA damage, associated with metformin treatment (Refs 112, 113).

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Table 2. A summary of therapies that influence cyst growth in ADPKD and their effect on DNA damage

Factor or system	Effect on kidney cyst growth	Refs	Effect on DNA damage	Refs
Pharmacological interventions				
ACEi or ARBs	Decrease[a]	102	Decrease[b,c]	83, 84, 103
Tolvaptan	Decrease[a]	104–106	Increase[b,c] Decrease[c]	↑ 107, 108 ↓109
Metformin	Decrease[b,c]	110, 111	Increase[b] Decrease[a,b,c]	↑ 112, 113 ↓ 114–120
Nicotinamide	Decrease[c]	121	Decrease[a,b,c]	122, 123
Curcumin	Decrease[c]	124	Increase[b] Decrease[c]	↑ 125–127 ↓ 128
Glucosylceramide synthase inhibitors	Decrease[c]	129	Unknown	N/A
Non-pharmacological interventions				
Dietary sodium intake	Increase[a]	130–132	Increase[b]	57, 82
Caloric restriction	Decrease[a,c]	133–135	Decrease[a,b,c]	136–139

ACEi, angiotensin converting enzyme inhibitors; ARBs, angiotensin II receptor blockers.
[a]Denotes this effect was demonstrated in human studies.
[b]Denotes this effect was demonstrated *in vitro*.
[c]Denotes this effect was demonstrated in animal studies.

Curcumin: Leonhard *et al.* revealed curcumin treatment was effective at reducing two kidney weight to body weight ratio, cystic index, proliferation and STAT3 activation in the iKsp-*Pkd1*^{del} mouse model (Ref. 124). The clinical efficacy of curcumin as an oral supplement (25 mg/kg per day) in treating vascular dysfunction in ADPKD is currently being investigated in a paediatric and young adult population (6–25 years of age) (ClinicalTrials.gov Identifier: NCT02494141; https://clinicaltrials.gov/ct2/show/study/NCT02494141). A secondary outcome measure for this study is change in urine 8-OHdG after 1 year of curcumin treatment, as a biomarker of oxidative stress. The effect of curcumin in DNA damage and DNA repair proteins has also been examined *in vitro* and *in vivo* (Refs 125, 126, 128, 141). *In vivo*, curcumin significantly improved survival rate of tumour-bearing mice (Ref. 128). *In vitro*, curcumin reduced DNA damage induced by carboplatin treatment (chemotherapeutic agent) and increased expression of DNA repair pathway proteins such as BRCA1, BRCA2 and ERCC1 (Ref. 128). In contrast, studies in mouse-rate hybrid retina ganglion N18 cells, demonstrated that curcumin treatment induced DNA damage and reduced expression of DNA repair proteins including ATM, ATR, *BRCA1* and DNA-PK (Ref. 141). Moreover, in human hepatoma G2 cells, curcumin increased 8-OHdG staining in a dose-dependent manner (Ref. 126). Owing to this conflicting evidence, Cao *et al.* proposed a dual, dose-dependent role for curcumin, where low level curcumin treatment exhibits antioxidant properties and high doses cause oxidative stress and DNA damage (Ref. 126).

Glycosphingolipid (GSL) metabolism: Altered GSL metabolism has emerged as a pathological feature of hyperplastic or hypertrophic renal diseases, including renal cell carcinoma, diabetic nephropathy and PKD, because of its role in regulating proliferation and apoptosis (Refs 142, 143). Elevated components of GSL metabolism including glucosylceramide, lactosylceramide and ganglioside GM3 have been reported in human ADPKD and the *cpk* mouse (Refs 144, 145), and Natoli *et al.* demonstrated that pharmacological inhibition of glucosylceramide synthase with Genz-123346 (blocks conversion of ceramide to glucosylceramide) reduced cystic disease burden and improved renal function in numerous mouse models of PKD (*Pkd1* conditional knockout mice, *jck* mice and *pcy* mice) via downregulation of Akt-mTOR pathways and inhibition of cell cycle (Ref. 129). Recruitment is currently underway for a 2-year intervention study (STAGED-PKD) to determine whether venglustat (a glucosylceramide synthase inhibitor) is effective at reducing the rate of total kidney volume growth and eGFR decline in ADPKD patients with rapidly progressive disease (18–50 years of age, eGFR 45–90 mL/min/1.73 m^2, Mayo Imaging Classification Class 1C-E; ClinicalTrials.gov Identifier: NCT03523728; https://clinicaltrials.gov/ct2/show/NCT03523728). Studies have demonstrated interaction between sphingolipids and the DDR, as reviewed by Carroll *et al.* (Ref. 146), where elevated ceramides may be a downstream consequence of ATM activation *in vitro* (Ref. 147), as well as associated with conditions of oxidative stress (Refs 148, 149). However, there is currently no evidence to suggest glucosylceramide synthase inhibition directly influences levels of DNA damage and/or DNA repair mechanisms.

In summary, almost all current therapies in use or under investigation for the treatment of ADPKD, at least in part, suppress DNA damage and/or oxidative stress. However, further studies are required to clarify the extent to which these therapies are mediated, at least in part, by their effect on suppressing DNA damage.

Conclusion and future directions

This review has outlined the current evidence linking DNA damage and kidney cyst formation in ADPKD. The proposed hypothesis is outlined in Figure 1b. Briefly, we propose that exposure to DNA-damaging events is causal in the formation of somatic mutations and reduced gene dosage, resulting in subsequent kidney cyst formation. This hypothesis requires further investigation in future studies, and specific questions that should be addressed include the following:

(i) *Verification that DNA damage is increased in ADPKD compared with healthy controls.* Future studies are required to examine the *in vivo* characteristics and kinetics of DNA damage and DDR signalling in genetically orthologous animal models and human ADPKD, in relation to the progression of cystic kidney disease. These studies will determine if the presence of DNA damage precedes or follows cyst formation in ADPKD, and whether DNA damage is increased in early- or late-stage disease.

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(ii) *Are ADPKD patients more susceptible to DNA damage?* Further investigation is required to examine whether *PKD1* deficiency results in increased susceptibility to DNA damage, and whether DNA repair is defective in ADPKD. This hypothesis can be examined *in vitro* and *in vivo* by exposing PKD models to DNA damaging agents (e.g. UV light, cytotoxic agents) and observing their response compared with healthy controls. Genomic analyses would also be useful to examine whether mutations in *PKD1* or *PKD2* are also associated with mutations in DDR genes, potentially providing an explanation for a defective DDR in ADPKD.

(iii) *Will modifying DNA damage influence disease outcomes in ADPKD?* It remains unknown whether reducing or increasing DNA damage will improve disease outcomes in ADPKD. Initial studies are required to confirm the functional significance of DNA damage. If DNA damage proves causal, studies would be required to examine if approaches to reduce DNA damage (e.g. antioxidants) are effective at reducing cyst burden in ADPKD. An examination of mutational signatures and patterns observed in cystic tissue compared with non-cystic tissue may be useful in identifying whether any changes in DDR occur as a cyst-initiating event. Furthermore, the use of a systems biology approach will be most useful to determine the therapeutically potential for modifying DNA damage in ADPKD. An examination of transcriptomics, proteomics and metabolomics can reveal novel pathways and mechanisms involved in disease pathogenesis, allowing for the discovery of new small molecule drug targets and drug re-purposing. Numerous 'omics' analyses have been conducted previously in PKD using cell lines, rat or mouse tissue and human samples (Ref. 150), and further examination of this data from a DNA damage and DDR perspective would be of interest.

ADPKD is a genetic condition that affects approximately 12 million people worldwide, where at least half will experience ESKD during their lifetime. Owing to the economic burden of renal replacement therapy and the increasing longevity of the population, the identification of drug targets and development of treatments to ameliorate kidney cyst formation in ADPKD are vital. Targeting DNA damage may provide a promising path forward towards curative ADPKD therapies.

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