# Current epigenetic aspects the clinical kidney researcher should embrace

**Authors:** Anna Witasp[1,2], Amaryllis H. Van Craenenbroeck[1,3], Paul G Shiels[4], Tomas J. Ekström[2,5], Peter Stenvinkel[1], Louise Nordfors[1,2,6]

**Affiliations:** [1]Division of Renal Medicine, Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden; [2]Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; [3]Department of Nephrology, Antwerp University Hospital & University of Antwerp, Antwerp, Belgium; [4]Institute of Cancer Sciences, MVLS, University of Glasgow, Scotland, UK; [5]Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden; [6]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden.

**Correspondence to:** Peter Stenvinkel, Division of Renal Medicine, Karolinska University Hospital at Huddinge M99, Karolinska Institutet, SE-14186, Stockholm, Sweden

Tel.: +46-8-58582532, Fax: +46-8-7114742

E-mail: peter.stenvinkel@ki.se

**Key terms: chronic kidney disease, epigenetics, DNA methylation, microRNA**

**Word count:** 6637

**Number of figures:** 1

**ABSTRACT**

Chronic kidney disease (CKD), affecting 10-12% of the world's adult population, is associated with a considerably elevated risk of serious comorbidities, in particular premature vascular disease and death. Although a wide spectrum of causative factors has been identified and/or suggested, there is still a large gap of knowledge regarding the underlying mechanisms and the complexity of the CKD phenotype. Epigenetic factors, which calibrate the cellular mechanisms for transcriptional regulation, are emerging as important players in the CKD-associated pathophysiology. In this article we review some of the current knowledge on epigenetic modifications and aspects on their role in the perturbed uraemic milieu, as well as the prospect of applying epigenotype-based diagnostics and preventive and therapeutic tools of clinical relevance to CKD patients. The practical realization of such a paradigm will require that researchers apply a holistic approach, including the full spectrum of the epigenetic landscape as well as the variability between and within tissues in the uraemic milieu.

**ABBREVIATIONS**

| | |
|---|---|
| AMPK | 5’ adenosine monophosphate-activated protein kinase |
| AT1R | angiotensin II type 1 receptor |
| CKD | chronic kidney disease |
| CLDN1 | claudin-1 |
| CRP | C-reactive protein |
| CVD | cardiovascular disease |
| DN | diabetic nephropathy |
| DNMT | DNA methyltransferase |
| ESRD | end-stage renal disease |
| FGF23 | fibroblast growth factor 23 |
| GWAS | genome-wide association study |
| 5hmC | 5-hydroxymethyl cytosine |
| HAT | histone acetyltransferase |
| HD | hemodialysis |
| HDAC | histone deacetylase |
| HDACi | histone deacetylase inhibitors 1 |
| HDM | histone demethylase |
| HMT | histone methyl transferase |
| IFNG | interferon gamma |
| IL-6 | interleukin 6 |
| lncRNA | long noncoding RNA |
| MBP | methylation binding protein |
| $m^6A$ | N6-methyladenosine |
| 5mC | 5-methyl cytosine |
| miRNA | microRNA |
| mtDNA | mitochondrial DNA |
| mTOR | mammalian target of rapamycin |
| PD1 | programmed cell death protein 1 |
| piRNA | piwi-interacting RNA |

| | |
|---|---|
| RNAi | RNA interference |
| RNMT | RNA methyltransferase |
| ROS | reactive oxygen species |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethionine |
| SASP | senescence associated secretory phenotype |
| SHC1 | SHC adaptor protein 1 |
| SUMO | small ubiquitin-like modifier |
| TGFB1 | transforming growth factor B1 |
| TNF | tumor necrosis factor |
| VSMC | vascular smooth muscle cell |

## INTRODUCTION

Chronic kidney disease (CKD) is a heterogeneous condition with a wide spectrum of functional and structural changes leading to successive loss of renal function over time [1]. The prevalence of CKD parallels an increased prevalence of type-2 diabetes, psychosocial stress, obesity and a sedentary life style [2]. Chronic kidney disease may thus be seen as a reflection of the ”burden of life style” where epigenetic alteration of gene expression act as a dynamic regulator of responses to change in environmental conditions [3]. Consequently, associations between the uraemic phenotype and the uraemic epigenotype deserve further research. An estimated 10-12% of the world’s adult population is affected by CKD and although a large proportion is distributed among the elderly, CKD exists across all age groups [4]. Regardless of age, patients with CKD face an imminent risk of either progressing to end-stage renal disease (ESRD) and/or developing cardiovascular disease (CVD); both associated with decreased survival [5]. The mortality risk is proportional to the decline in renal function and whereas it is diminished by renal transplantation, dialysis provides only a modest risk reduction and mortality risk is actually higher soon after initiation of hemodialysis (HD) [6]. Dialysis patients display an annual mortality rate of 20%, which is comparable to metastatic colon and ovarian cancer [7], and the risk of death due to CKD-associated CVD is more than 20-fold compared to the general population [5]. Even patients with early-stage CKD have a dramatically increased risk of mortality and most patients will die before they reach the terminal stage of the disease [5]. Why CKD is associated with such a poor prognosis and large inter-individual variation with respect to comorbidities, rate of disease progression and survival [8], is still not well understood. This is reflected by the paucity of established disease biomarkers and efficient treatment options. The underlying mechanisms remain incompletely understood, though it is likely that both genetic and epigenetic factors contribute to the observed variability.

Recent developments in genomic technologies have offered significant opportunities to extract novel information in an unbiased manner, which may help identify individuals at higher risk of developing CKD and its associated complications. Indeed, the nephrology community has put a lot of faith and effort into multi-consortia genome-wide association studies (GWASs) to illuminate common genetic variants in potential pathogenic pathways [9-16]. GWAS-extracted gene candidates have, however, hitherto not given an explanation to the considerable variability observed between CKD patients [17]. This “missing heritability” may partly be due to unidentified rare variants, but also gene-environment interactions and acquired epigenetic changes [18]. Epigenetic changes are dynamic and

influenced by external and internal environmental cues [19, 20], unlike the genetic information, which is "locked" in the primary genomic sequence from birth (disregarding spontaneous mutations). This is important, as epigenetic regulation of the genome allows genes to become actively expressed or repressed, which may in turn translate into different phenotypic traits. Any change in epigenetic regulation that deviates from a healthy state may therefore be of functional importance in disease development [21, 22]. Knowledge of epigenetic changes in human disease has expanded [23, 24]. In this review, we will provide an updated summary of some basic concepts of epigenetic modifications and elaborate upon their role in the uraemic milieu, in particular how these changes relate to premature vascular disease. Although the literature remains sparse, epigenetic changes as potentially modifiable targets in the treatment of CKD will also be discussed.

## EPIGENETIC MODIFICATIONS

Epigenetic modifications are, by definition, stably heritable (through replication and cell division) traits which modulate the physical structure of DNA, without affecting the primary nucleotide sequence [25].

These modifications may act transiently, or persistently, as a temporal cellular memory over time, to provide means for diversified gene expression programs and phenotypes in different cell types in multicellular organisms. Such spatio-temporal specificity is integral to a range of cellular developmental processes (e.g. cell differentiation and cell maturation during early development) as well as homeostatic processes. The most well-studied epigenetic mechanisms include covalent posttranslational modifications of histones and DNA cytosine methylation, activities which are closely allied with non-coding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs).

### Histone modifications

In the human cell nucleus, DNA exists in tightly folded and organized nucleosomal structures, primarily complexed with histone proteins, of which H2A, H2B, H3 and H4 are the most common. These proteins are assembled as octamers, each with a segment of DNA coiled around it, forming the nucleosomes, which are packaged into chromatin macromolecules. The nucleosome allows coordination of the physical structure mainly through its extruding N-terminal ends, but also via their C-terminal regions, where histone tails have amino acid residues that are accessible for enzymatic modifications. Well-characterized histone marks

include trimethylation (e.g. H3K27me3, H3K4me3 and H3K36me3), demethylation, acetylation (e.g. H3K27ac, H3K14ac), ubiquitination and SUMOylation of lysine residues, as well as methylation of arginines and phosphorylation of serine residues [26, 27]. These enzymatic modifications are, among others, mediated via specific histone methyl transferases (HMTs) and demethylases (HDMs), histone acetyltransferases (HATs) and deacetylases (HDACs, including sirtuins), ubiquitin ligases and de-ubiquitinases, small ubiquitin-like modifier (SUMO) ligases and proteases, and kinases and phosphatases.

Histone modifications regulate gene activity by making the chromatin more or less condensed, and hence more or less accessible for transcription factors, but also by defining chromatin structure and its temporal and spatial context in the nucleus [28]. As an example, acetylation of amino acid residues in the histone tail often propagate a less condensed, transcriptionally active state [29], while methylation at the same or other sites may correlate with a more condensed, transcriptionally inert, structure. In addition to regulating gene activity, histone modifications are crucial for other biological processes, such as initiating DNA replication by dimethylation of H3K79, and chromosome condensation during mitosis, which is mediated by a cascade of histone modifications, including phosphorylation of H3S10 by the Aurora B kinase [30, 31]. Histone modifications also play a vital role in the DNA repair process by marking sites of DNA damage; as an example gamma-H2AX is extensively phosphorylated in response to double strand breaks [32, 33]. The integration of all possible combinations of different histone tail sites with different modification provides an enormous complexity to the epigenetic regulation, sometimes referred to as a histone code [26]. The complexity becomes even greater when adding the close cross-talk existing between histone modifications, DNA methylation and non-coding RNAs (e.g. reviewed in [34]).

Chromosomes are also modulated at a higher level. Chromatin remodeling, i.e how chromatin is folded and organized within the nucleus, is a rapidly growing field of research, as its implications in development and disease are increasingly recognized. Recent development of chromosome conformation capture (3C) technologies has made high resolution mapping and visualization of 3D chromatin architecture possible [35], but has at the same time shown that the mechanisms behind chromatin organization are substantially more complex than first anticipated [36]. To regulate gene activity and other key biological processes, several components, such as transcription factors, architectural proteins and ncRNAs, collaborate to modulate the chromatin architecture at different levels. For example, chromatin loops are formed to enable long-distance enhancer-promoter interactions [36] and at a higher level,

chromosomes are being directed to distinct territories within the nuclear space. Mutations in genes encoding ATP-dependent chromatin remodelling enzymes, often referred to as Snf2- or SWI/SNF-related enzymes, occur at a high frequency in many cancers and have also been associated with a number of complex diseases (reviewed by [37]). For an extensive review on the organization and function of the 3D genome see [38].

**DNA methylation**

Active DNA methylation and demethylation have important functions in normal and pathological cellular processes and contribute to development and differentiation of cells and tissues, including tissue-specific gene expression, genomic imprinting, silencing of retroviral elements and X chromosome inactivation [39-41]. DNA methylation involves the addition of methyl groups onto the 5′-position of cytosine (5mC) rings by DNA methyltransferases (DNMTs) and occurs either to maintain original methylation patterns during replication, or to produce *de novo* DNA methylation. One effect is gene transcription silencing via recruitment of repressor proteins or prevention of transcription factor binding to DNA [34].

DNA methylation generally appears within the context of CpG dinucleotides. When CpGs become methylated, methylation-binding proteins (MBPs) that bind to the site generate a transcriptional repressive mode via recruitment of chromatin remodeling factors (including HDACs). Most CpG sites within the mammalian genome are methylated, including CpGs found in and between genes, where intergenic DNA methylation is known to play an important role to repress transcription of transposable and viral elements or other potentially harmful genetic elements. The function of gene body DNA methylation is however, still unclear [42]. In contrast, CpG islands, i.e. regions of approximately 1000 base pairs that are enriched for CpG sites, are commonly depleted of methylated DNA, allowing an open chromatin structure and binding of transcription factors. CpG islands are highly conserved between mice and humans [43] and approximately 70% of all gene promoters, in particular promoters for housekeeping genes, are found in CpG islands [44]. In addition, conserved regions called CpG island shores, located up to 2 kb from CpG islands, show tissue-specific methylation patterns, which correlate with reduced gene expression [45]. The effects of DNA methylation on the genome may be counteracted by DNA demethylation reactions [46]. 5-hydroxymethyl cytosine (5hmC) is the first oxidative product in this process [46], shown to correlate with active gene transcription [47, 48]. As commonly utilized methods for detecting DNA methylation, including sodium bisulfite sequencing, do not discriminate between 5mC and 5hmC, all reports on 5mC assessed with bisulfite-mediated methods may be confounded by the potential presence

of 5hmC. Thus, new methods such as oxidative bisulfite sequencing have been developed to allow discrimination between the two [49].

**RNA methylation**

RNA methylation is the key component of the epitranscriptome. Although the phenomenon has been known for over 60 years [50], it remains one of the least understood aspects of epigenetic regulation. RNA methylation is found in a wide range of RNA species (including transfer RNA, ribosomal RNA, messenger RNA, transfer-messenger RNA, small nuclear RNA, small nucleolar RNA, miRNA, and viral RNA) as the result of the activities of distinct RNA-methyltransferases (RNMTs) [51]. The best characterized eukaryotic methylation of RNA is N6-methyladenosine ($m^6A$), although 5mC is common. Recent data suggest that $m^6A$ and 5mC RNA methylation affect the regulation of a plethora of metabolic processes, including RNA stability and translation, and that aberrant RNA methylation contributes to the aetiology of human disease [52]. Recent discoveries have shed new light on the importance of $m^6A$ mRNA methylation for the epitranscriptomic regulation of circadian clock genes [53] and the subsequent impact on metabolism. Fustin and co-workers have revealed that internal $m^6A$ RNA methylation of clock gene transcripts is a key regulator of circadian rhythm and that period length is inversely proportional to the methylation potential. They have also shown that the circadian cycle is dramatically lengthened following inhibition of $m^6A$ methylation by siRNA-mediated knockdown of the $m^6A$ methylase *Mettl3* and that it is shortened by *Mettle3* overexpression.

RNA methylation has been postulated as a basis for epigenetic transcriptional memory in response to a previous stimulus, which can produce heritable changes in the response of an organism to that stimulus. A sequitur from this would be a quantitative, or qualitative change in gene expression. Significantly, direct evidence for this lies in chromatin changes regulating binding of RNAPII, which are conserved across taxa [54]. A role for RNA methylation has yet to be established in renal disease, though one immediate area it may manifest in is immune function, where post transcriptional regulation of T cell memory (and by extension transcriptional memory) by T cell miRNAs and methylation may be dysregulated. Precedence for this has already been established in SLE, where it contributes to tissue damage and autoantibody formation [55].

**Adenosine-to-Inosine RNA editing**

Another important post-transcriptional process is RNA editing, which has the ability to alter the nucleotide sequence of both coding and non-coding RNAs and is a widespread phenomenon occurring in more than half of the human transcriptome [56, 57]. The most common form of editing in mammals is adenosine-to-inosine (A-to-I) conversion and may either modify codons, or insert or remove splice sites, and hence alter the amino acid sequence or length of a protein. As inosines have properties mimicking those of guanosines, A-to-I editing may also disturb RNA-RNA base pairing and change the secondary structure of a transcript [58]. In non-coding RNAs, such as miRNAs that act by binding to other RNA targets, changes of the nucleotide sequence may also have implications on binding specificity. The A-to-I conversion is catalyzed by enzymes of the adenosine deaminase acting on RNA (ADAR) family, which are essential for normal development [56, 59] and abnormal ADAR activity has been associated with many human diseases including vascular disease [60], cancer, neurological disorders, metabolic diseases, viral infections and autoimmune disorders [61]. Drugs targeting the ADAR family of enzymes may seem as a promising way to future therapies. However, as the magnitude of RNA editing sites in the human genome has only recently been understood, and knowledge about the biological processes where ADARs are involved is limited, unwanted side effects using this approach may be considerable. An alternative strategy could be to target specific ADAR substrates, which was demonstrated by Tariq and co-workers, who managed to repress ADAR activity by altering RNA editing in a substrate-specific manner [62].

## Non-coding RNAs

### *miRNA*

The small (20-24 nucleotides) non-coding miRNAs add another layer of complexity to the regulation of gene expression that can have profound effects on biological pathways [63]. The miRNAs are an evolutionary conserved class of single-stranded, non-coding RNAs that regulate gene expression at the posttranscriptional level. As part of the RNA interference (RNAi) system, miRNAs merely have a negative regulatory function, either by degradation of target mRNA by targeting its 3' untranslated region and/or by inhibition of protein translation [64]. To date, over 1800 miRNAs have been identified in the human genome (Reference: www.miRbase.org), and they are estimated to affect the expression of more than 50% of the protein-coding portion of the human genome [65-67]. The miRNA coding sequences typically exist in intergenic areas but can also be found in sense or antisense orientation within introns

of genes. Some miRNAs are clustered in the genome and are most likely to be transcribed together [68].

One specific target gene can be regulated by many different miRNAs, and one single miRNA may alter the expression of a large number of target genes, often involved at different levels in a signaling cascade of a particular biological pathway. Indeed, the majority of miRNAs exert their effects through the rather modest reduction of a large number of targets which altogether give alterations in cellular phenotype [65]. Thus, most miRNAs are highly pleiotropic and act differently depending on the cell type. On the other hand, the regulation of miRNAs can be conducted via multiple steps, such as gene transcription, processing, transport and target recognition. miRNAs are also regulated by long non-coding RNA (lncRNA) [69] and mRNA targets can reciprocally control the level and function of miRNAs [67].

In addition, both histone modification and methylation status of miRNA promoters influence miRNA expression and action [70, 71]. As part of this epigenetic-miRNA regulatory circuit, miRNAs may in turn induce DNA methylation [72] and control the expression of DNMTs and HDACs [73], thereby altering gene transcription at a higher level. Adding to the complexity of miRNA regulation is also that, for many miRNAs, there are several length and/or sequence isoforms, termed isomiRs [74], that differ either in the 5'- or 3' ends (5' isomiRs and 3' isomiRs, respectively), or harbour internal nucleotide substitutions (polymorphic isomiRs) as compared to the canonical sequence. The polymorphic isomiRs are often a result of A-to-I RNA editing [75] and may alter target specificity if located in a seed region, i.e the sequence essential for the binding of the miRNA to the mRNA. Several such alterations have been implicated in human disease.

*lncRNA and piwi-interacting RNA (piRNA)*

lncRNAs comprise non-coding RNA species over 200 nucleotides long. They are both inter- and intragenically derived and show differential tissue specific expression [76]. These RNAs differ from miRNAs because they regulate gene expression not only at the post-transcriptional but also at the transcriptional level, as well as in post-transcriptional processing of miRNA, imprinting and DNA methylation, chromatin remodeling, cellular reprogramming, intracellular trafficking and cellular stress and damage responses [77].

Another class of small non-coding RNA molecules, the 26–31 nucleotides long piRNAs, also contributes to the non-coding RNA regulatory circuit in animal cells [78]. An important role

for piRNAs seems to be gene silencing of transposons via recruitment of DNA methyltransferases to transposable elements. [79]. The understanding of the RNA epitranscriptome and the function of non-coding RNAs in different cell types and states will likely improve rapidly as the development of single-cell RNA sequencing techniques advance [80]. For a comprehensive review on e.g. the functional role of lncRNAs in the kidney, see Lorenzen and Thum [81].

## DRIVERS OF EPIGENETIC CHANGES IN THE URAEMIC MILIEU

Decreased renal function generates a toxic internal milieu typified by hyperhomocysteinemia [82], chronic inflammation [83], oxidative and glycosative stress [84], gut dysbiosis [85], hyperphosphataemia [86] and dyslipidaemia [87] that increases the risk of adverse outcomes, including premature vascular ageing and CVD [88, 89]. The uraemic milieu may be linked to alterations in epigenetic regulation of cellular and physiological homeostasis [90, 91], **Figure 1.**

### Hyperhomocysteinemia

Homocysteine metabolism involves conversion of homocysteine to S-adenosylhomocysteine (SAH) via the derivatives methionine and S-adenosylmethionine (SAM). Whereas the latter serves as a universal methyl-group donor in many different methylation reactions, including DNA methylation, SAH is a competitive inhibitor of methyltransferases and may, thus, repress methylation reactions. In CKD, circulating homocysteine levels are markedly elevated [82] and predict cardiovascular outcome [92]. Although the homocysteine metabolism pathway is reversible, hyperhomocysteinemia favour SAH formation. Therefore, an accumulation of homocysteine is associated with DNA hypomethylation [93]. In line with this, studies on hyperhomocysteinemia in HD [94] or patients with CVD [95] show that those with elevated levels of SAH have a higher degree of global DNA hypomethylation compared to controls. Indeed, folate therapy, which lower homocysteine, partly restored DNA methylation in HD patients [94]. Yet others have conducted global DNA methylation analyses on CKD stage 2-4 patients and observed no associations between global DNA methylation and either homocysteine or carotid intima-media thickness [96]. As the discrepancy between these studies is likely due to differences in study design and methodology for DNA methylation assessment, it is possible that currently improved techniques will explain these paradoxical observations. Moreover, SAH, rather than homocysteine *per se*, has been suggested as the main culprit in

hyperhomocysteinemia-associated CVD risk [97]. Recent data suggest that SAH, through DNA hypomethylation, may mediate an enhanced pro-inflammatory state with elevations in intermediate monocyte cell counts [98]. Whether stimulation of DNA hypermethylation in monocyte subsets (by supplementation of SAM) will generate reduced intermediate monocyte cell counts remains to be proven.

**Inflammation**

Many CKD patients display persistently raised levels of inflammatory biomarkers, e.g. C-reactive protein (CRP), tumor necrosis factor (TNF) and interleukin 6 (IL-6), [99], which are associated with oxidative stress, protein energy wasting, endothelial dysfunction and vascular calcification, and serve as predictors of outcome [99]. Pro-inflammatory cytokines trigger changes in chromatin structure [100], regulate expression of DNMTs [101] and induce hypermethylation *in vitro* [91]. Similar to what has been observed in non-CKD populations [102, 103], chronic inflammation associates with DNA hypermethylation in CKD stage 3-5 and HD patients [91]. In addition, longitudinal observations have shown associations between both DNA hypermethylation and all-cause and CVD-mortality in incident dialysis patients [91]. However, the observed inflammation-associated hypermethylation may be a cell-specific phenomenon. Like other pro-inflammatory conditions, CKD is associated with a pronounced shift in the monocyte subset distribution, from classical towards intermediate and non-classical monocytes, and increased intermediate monocyte counts have been shown to predict cardiovascular events in CKD [104, 105]. Notably, intermediate monocytes are enriched for hypo- rather than hypermethylated loci compared to classical and non-classical monocytes [98]. This is probably also true in CKD as uraemic serum stimulates haematopoietic stem cells into a larger fraction of intermediate monocytes, as compared to control conditions, with an increased number of hypomethylated loci [98]. As several of these differentially methylated loci are linked to CVD, infections and/or immune diseases a potential link between uraemic toxins and reprogramming of monocytes into a pro-inflammatory cell profile via DNA methylation changes seems likely.

Recent evidence also points to a role of miRNA dysregulation in impaired monocyte subset differentiation in dialysis patients receiving intravenous iron therapy [106]. Indeed, the uraemic environment per se is able to alter miRNA profiles involved in inflammatory pathways [107]. In general, total plasma levels of small RNA, including miRNA, were shown to be significantly lower in severe CKD, possibly due to increased degradation [108, 109]. In spite of this

observation, circulating levels of particular miRNAs, e.g. miR-150, miR-143, miR-145, and miR-223, were reported to be significantly higher in CKD patients compared with healthy subjects, illustrating the specificity of miRNA up- and down-regulation [110]

**Oxidative stress**

Oxidative stress, a redox imbalance where the formation of reactive oxygen species (ROS) is increased and/or ROS degradation by the antioxidant system is reduced, is a prominent feature of CKD linked to a variety of disorders, including atherosclerosis and fibrosis [84]. Epigenetic marks may be implicated in this pathogenic association. For instance, the p66Shc gene (*SHC1*), encoding the p66Shc stress-response protein implicated in ROS metabolism, has been shown to be hypomethylated in dialysis patients, which may result in increased levels of p66Shc and risk for oxidative stress-mediated arteriosclerosis [111]. Oxidative stress is linked to epigenetic changes and active gene transcription via 5hmC [112], possibly in association with inflammation [113]. On the other hand, ROS recruit DNMT1 and SIRT1, associated with repression of gene transcription, to CpG islands [114]. Increased oxidative stress also decreases the DNA hydroxymethylome *in vitro* and *in vivo* [115]. Considering the role of hmC as an intermediate in demethylation reactions, this would intuitively imply that oxidative stress prevents hypomethylation and promotes hypermethylation on a global level. This may affect specific genes differently depending on their regulation by methylation. Considering the contrasting data on methylation in CKD, controlling for oxidative stress and DNA hydroxymethylation may be a way to reconcile these reports.

**Dyslipidaemia**

Significant alteration in lipoprotein metabolism is a typical feature of the uraemic milieu and is associated with increased cardiovascular risk [87]. The effect of hypercholesterolemia on chromatin structure has been documented for over 30 years [116] and studies made both in apolipoprotein E null mutant mice and on human monocyte cell lines have demonstrated a link between dyslipidemia and DNA methylation changes [117]. Data generated from two large cohort studies, the REGICOR study (discovery cohort) and Framingham Offspring Study (validation cohort), has identified associations between serum lipid profiles, including cholesterol, HDL-cholesterol and triglycerides, and differential DNA methylation on nine genes (SREBF1, SREBF2, PHOSPHO1, SYNGAP1, ABCG1, CPT1A, MYLIP, TXNIP, SLC7A11) and two intergenic regions [118]. Some of these findings, e.g. SREBF1, ABCG1 and CPT1A, are consistent with previous studies on plasma lipid levels in relation to DNA

methylation, using both global, candidate gene and whole-genome approaches, recently reviewed by Braun *et al* [119]. In contrast, the relationship between histone modifications and blood lipids is not well studied, as is the impact of uraemic dyslipidemia on epigenetic changes. Overall, its impact on the epigenetic landscape remains to be defined in any significant detail in relation to CKD.

**Hyperphosphataemia**

Hyperphosphatemia and disturbances in bone-mineral metabolism including the fibroblast growth factor 23 (FGF-23)/klotho axis are frequent features of the uraemic phenotype that are associated with premature vascular aging [88]. The renal klotho protein is down-regulated in the toxic uraemic milieu [120] and recent evidence suggest that uraemic toxins may affect *KL* expression via effects on its associated epigenotype. Sun *et al* [121] reported that inhibition of *KL* gene expression by uraemic toxins correlates with gene hypermethylation. Since this study shows that accumulation of uraemic toxins can silence *KL* expression via hypermethylation, this opens a route for the epigenetic targeting of specific genes. Indeed, Rhein (a compound isolated from rhubarb with known renoprotective effects) was recently reported to reverse *KL* repression via promoter demethylation and protected mice with CKD against kidney and bone injuries [122] Thus, targeting the epigenome may have therapeutic implications for disease states associated with premature aging and low *KL* expression. Whether hyperphosphataemia and premature vascular calcification are related to changes in the epigenome remains unproven and controversial. Montes de Oca *et al* [123] have shown that high phosphate levels induce methylation of the smooth muscle cell-specific protein SM22α promoter and loss of its activity. Since this was accompanied by calcification, increased alkaline phosphate activity and gain of the osteoblast transcription factor Cbfa1, it can be speculated that high phosphate levels, at least in part, promote vascular calcification via epigenetic effects. On the other hand, Uchiyama *et al* [124] reported that although a phosphate-rich diet caused hypermethylation of the vitamin D receptor-, and calcium-sensing receptor genes in rat parathyroid glands, the degree of hypermethylation was of such a low magnitude that it was insufficient to down-regulate gene expression. Thus, the effects of a high phosphate diet on the epigenotype need further studies.

Experimental data are supportive of a direct effect of hyperphosphatemia on the expression of specific miRNA. In cell culture models of human vascular smooth muscle cells (VSMCs), the addition of phosphate resulted in a decreased expression of miR-30b, miR-133 and miR-143; miRs targeting Smad1 and Osx [125]. Similarly, both *in vitro* (VSMC) and *in vivo* (rodent model of CKD), with high Pi inducing lower expression levels of miR-133b and

miR-211 (targeting *RUNX2*) and upregulation of miR-29 [126]. Functional experiments modifying these specific miRNAs further confirmed their regulating role in the calcification process.

### Gut dysbiosis

The link between epigenetic mechanisms in the human microbiota and how it affects human disease is under intense investigation [127]. Various metabolites from the metabolically active biomass of gut microbiota can interact with the mammalian epigenetic machinery, including histone modifications and DNA methylation [128]. It is of interest that CKD patients display a distinct gut microbial metabolism [129] and gut microbiota is associated with increased blood pressure in animal models [130]. Moreover, decreasing the gut microbiota biomass by antibiotics affect blood pressure [131]. However, the consequences of gut dysbiosis and effects on epigenetic modifications in the context of CKD are not clear and, thus, the possibility of using therapeutics targeting gut microbiota, e.g. probiotics, and epigenetic changes in patients with CKD remains to be examined.

## EPIGENETIC CHANGES: IMPACT ON PHENOTYPE AND FUNCTION

Epigenetics research, including maladaptive regulation of cellular programming, memory and adaptations in relation to human disease, has taken a great leap forward during the past decade and the role of epigenetics has been elucidated in a number of human diseases such as cancer [132], psychiatric and neurological disorders [34], diabetes mellitus [133], rheumatoid arthritis [134] and atherosclerosis [135]. Advances in technologies for chromatin immunoprecipitation sequencing, genome-wide DNA methylation analyses and RNA sequencing have facilitated this development. The impact of uraemia on epigenetic changes and subsequent genomic dysregulation, is still a rather novel field. In the sections below epigenetic studies that relates to CKD phenotype are discussed.

### Epigenetics in CKD and disease progression

*Epigenetic dysregulation in renal physiology and disease progression*

A growing number of studies have revealed the involvement of miRNAs in the development and progression of both CKD and CVD [136, 137]. MicroRNAs play a critical role in renal physiology, including kidney development and function [138]. Loss of miRNA by selective deletion of the miRNA-processing enzyme Dicer in mouse podocytes leads to severe

glomerulopathy, proteinuria and loss of renal function [139, 140]. Epigenetic aberrations may also play a role in renal fibrosis, a hallmark of kidney disease progression. Transforming growth factor B1 (TGFB1) is a major regulator of renal fibrosis, and its signaling is tightly regulated by the expression of many specific miRNAs [138]. Moreover, particular miRNAs modulate the systemic, as well as intra-renal inflammatory response [141]. Inflammation and fibrosis are mediated by Smad3 signaling, which has been shown to interact with lncRNAs, including *Arid2* [81] and DNA methylation changes may also contribute. A genome-wide DNA methylation study of human renal tubule samples from healthy subjects and CKD patients demonstrated an enrichment of methylation changes for several genes known to be related to kidney fibrosis, including genes encoding collagens. These changes also correlated with downstream transcription levels [142]. Altogether, there is a potential role for an epigenetically dysregulated gene transcription of core pro-fibrotic pathways involved in CKD development.

*Epigenetic changes in diabetic nephropathy (DN)*

Epigenetic changes are clearly implicated in pathogenic processes causing DN [143]. For instance, studies on diabetic mice indicate that histone modifications and DNA methylation have protective effects on kidney damage and diabetic kidney disease via Sirt1 deacetylase activity [144]. Deacetylation of histone H3 and H4 triggers subsequent CpG methylation of the Claudin-1 (*Cldn1*) gene, and lower *SIRT1* and higher *CLDN1* expression in proximal tubule and glomerular regions were associated with heavy proteinuria in patients with DN [144]. Other studies suggest that SIRT1 maintains actin cytoskeleton in damaged podocytes via deacetylation of cortactin [145]. In addition, altered DNA methylation patterns causes mRNA expression changes in the proximal tubules in diabetic mice [146] and aberrant expression of several miRNAs, including miR-29a/b/c, Let-7b, miR200a and miR-21 seem to modulate pro-fibrotic pathways associated with DN [147-150].

*DNA methylation in the uraemic milieu*

Pioneering analyses made on global DNA methylation changes in CKD patients showed both DNA hypo- [94, 111] and hypermethylation [91], but without specifying any loci. More recent genome-wide analyses with single-nucleotide resolution have identified the precise locations of differentially methylated loci associated with CKD. One of these DNA methylation profiling studies, using the Illumina HumanMetylation27 Bead Chip Array, compared saliva-extracted DNA from diabetes patients with and without DN and were able to pinpoint 187 differentially methylated genes [151]. About 20% of these genes were previously reported to be involved in

kidney development, DN or dialysis-induced changes. Smyth *et al* [152] evaluated whole-blood DNA from 255 patients with CKD and 152 individuals without evidence of CKD using the larger array Illumina Infinium HumanMethylation450 BeadChip. Twenty-three genes showed significant methylation changes associated with CKD of which the strongest biological candidates included *CUX1, ELMO1, FKBP5, INHBA-AS1, PTPRN2* and *PRKAG2*. Two of these, *ELMO1* and *PRKAG2*, also showed altered gene transcription.

## Premature vascular ageing

### *Accelerated biological ageing and the epigenetic clock*

As a prematurely aged phenotype is a prominent feature of CKD [88, 89] clarification of how factors in the uraemic milieu affect the epigenetic clock and age-associated loci deserve attention [153]. Cellular attrition occurs at genetic and epigenetic levels during ageing of healthy individuals. The epigenetic landscape of normal ageing is featured by a general pattern of global DNA hypomethylation [19, 23, 154] and DNA damage-induced loss of heterochromatin [155]. In human kidneys, miRNA regulation of biological age and health span has been associated with expression levels of miRs 125a-5p, 125b and 217 [156]. These in turn are regulated by DNMT activity at their coding loci [157, 158]. This cross-talk is believed to be both central for enabling adaptation of renal physiology via changes in cellular metabolism and to its dysregulation in a uraemic milieu, where changes in the cellular methylome will have a direct effect of the activity of these miRNAs and hence age related renal function. Ageing is characterized by both inflammation and oxidative stress as well as a gradual change in the DNA methylome, which lead to increased methylation within the CpG islands and a loss of methylation at sites outside [23, 154]. This genome-wide dysregulation of DNA methylation patterns correlates with chronological age in various tissues [159-164] and changes in gene expression [165, 166]. Importantly, the identification of site-specific DNA methylation ageing-patterns spurred researchers to construct quantitative models to predict age [165, 167-169], which were recently used as a tool to study the relationship between estimated epigenetic age and disease. Individuals with a higher epigenetic than chronological age were at increased risk for all-cause mortality [169] and an elevated hepatic epigenetic age strongly correlated with a high body mass index [170]. The relation between epigenetic and chronological age in CKD has not been studied, but should be highly relevant due to the premature ageing phenotype typical for CKD [88, 89]. The underlying mechanisms are unclear, but a plausible explanation is that the toxic uraemic milieu impairs regulation of the ageing process via inflammation,

resulting in increased cellular senescence, activation of the senescence associated secretory phenotype (SASP), oxidative stress and telomere attrition and/or decreased expression of anti-ageing factors [89, 171]. These pathways are likely mediated, at least partly, via epigenetic regulation of ageing processes. Telomere length, which is linked to ageing and stem cell dysfunction, is regulated by histone modifications and DNA methylation [172]. As telomere attrition has been observed following dialysis initiation [173] and associate with mortality in prevalent HD patients [174] the possibility of an epigenetic dysregulation of telomere length in uraemia deserves further attention.

*Vascular disease*

Premature vascular ageing in CKD patients is an example of segmental ageing; i.e. the biological age of organs in the same individual differ markedly [175], probably as a result of tissue-specific epigenetic changes. Emerging data infer strong links between epigenetic dysregulation and individual susceptibility to CVD [22] and dysregulated DNA methylation patterns of human atherosclerosis-related genes have been reported in HD patients [176]. In mice, the first evidence for the involvement of miRNAs in vascular biology has come from studies showing that deletion of the Dicer enzyme resulted in impaired blood vessel and yolk sac formation [177]. The function of Dicer in endothelial cells was confirmed by silencing Dicer expression in cultured endothelial cells, which resulted in angiogenesis defects [178]. Until now, different miRNAs have been shown to be involved in vascular homeostasis and pathophysiology, including (not exhaustive list) miR-21, miR-34a, miR-126, miR-146a, miR-210 and miR-150 [179-181]. In CKD, different miRNAs are described to be involved in vascular disease in HD patients and in a rat model of CKD, including miR-21, miR-34a, and miR-126 [182, 183] [184]. Interestingly, miRNAs also play a role in blood pressure control and the renal and cardioprotective effects of RAAS blockade are at least partially mediated by specific miRNAs [185, 186].

## THE SEARCH FOR NOVEL BIOMARKERS IN CKD

It is important to realize that there are a number of limitations associated with global epigenetic profiling studies and that such results must be interpreted with caution. One limitation is that the epigenome often differs between tissues. Thus, the methylation pattern of blood derived DNA may not be reflecting the pattern of e.g. the varying cell types in renal tissue. Other

limitations may be associated with the analysis method, or depend on insufficient sample size and lack of detailed phenotype data. In addition, many studies are confounded by reverse causality and it is often hard to distinguish causation from correlation.

Despite intense research, novel biomarkers that perform better and are more specific and cost-effective than kidney biopsy and established biomarkers such as S-creatinine, and albuminuria are still lacking [187]. Deciphering the role of epigenetic modifications and miRNA in CKD disease progression and complications may be one feasible way to find improved prognostic biomarkers and novel therapeutic approaches. Recent findings on methylation sites associated with DN [151], CKD [152] and CKD-associated CVD [176] have suggested candidate genes that may be evaluated as predictive biomarkers of disease susceptibility and prognosis. In ANCA-associated vasculitis, gene-specific DNA methylation changes have been shown to predict remission [188]. On the other hand, as these studies were conducted on blood-derived DNA, the reported loci do not necessarily reflect causal mechanisms due to tissue-to-tissue and even cell variations of epigenetic patterns. To reveal pathogenic mechanisms with biological relevance, findings need to be considered in relation to their presumed site of action. Importantly, a large fraction of the candidates reported by Smyth *et al* [152] seemed to overlap findings in the kidney [142], suggesting the potential of using these as clinically useful biomarkers for CKD development. Indeed, it is vital that data from tissue analyses are mirrored by similar changes in blood or urine, or other less invasive tissues before they can become relevant for clinical purpose. In this regard, miRNAs are of particular interest as they act intracellularly but are present in body fluids (including plasma, serum and urine) [189] and show remarkable stability and resistance to degradation [190]. miRNAs are actively secreted and carry genetic information from one cell to another [137, 191] in extracellular vesicles (including microvesicles, microparticles, apoptotic bodies, exosome like vesicles and exosomes) that physically shield miRNAs from endogenous RNAse activity [192, 193]. Thus, a specific miRNA signature is now defined with high sensitivity for the development of albuminuria in patients with DN within two years [194, 195]. In autosomal dominant polycystic kidney disease, the pathogenic role of miR -21 and the miR-17~92 cluster is gradually being unraveled [196, 197]. Similarly, the fibromiRs miR-21, miR-214 and miR-199a are thought to be involved in the disease progression of IgA nephropathy [198].

## EPIGENETIC CHANGES AS THERAPEUTIC TARGETS IN CKD

The prospect of manipulating the epigenome, or rather specific loci, is of clinical interest since this would mean targeting specific gene regulation that could alter pathological pathways and decrease disease activity. Although this field remains immature within nephrology, emerging observations, both in CKD and other chronic diseases, such as cancer [27], hold promises for finding novel means to delay, inhibit or reverse CKD progression as well as its complications. Such therapeutics include pharmaceutical targeting of epigenetic changes, miRNA-based therapeutics and lifestyle interventions. However, it needs to be emphasized that there is a multi-faceted challenge when identifying and using epigenetic modifiers, as the epigenetic landscape varies between tissues and cell populations, and drugs that target histone modifiers will also have effects on non-histone proteins, together increasing the risk of unwanted side effects.

### Epigenetic drugs

A number of drugs are currently available that target epigenetic modifiers, in particular histone modifiers. We will not go into specific drugs but rather discuss principles. Most drugs targeting the epigenome were designed to repress carcinogenesis and can be divided into two classes: histone modifiers and DNA methylation inhibitors [23, 199]. Histone modifications involve both writers and erasers, i.e. enzymes that add modifications to specific amino acid residues, and that remove them, respectively. There are for instance both acetyltransferases and deacetylases that primarily modify lysines in histone (and non-histone) proteins, which can be inhibited by drugs. This may be used to fine-tune overall acetylation levels. The homeostasis of specific histone amino acid residue methylation is in turn regulated by methyl transferases and demethylases for which there are specific inhibitors. Other modifications may be specifically inhibited, such as phosphorylation/dephosphorylation of serine and threonine, but their specificity for histone kinases and phosphatases appear to be lower.

Bromo and chromo domain proteins are readers, which bind to acetylated and methylated histones tails, respectively, conferring biological effects by recruiting other proteins into regulatory complexes. Also here, a number of probes can be obtained that inhibits function. Although epigenetic drugs are still in their infancy, histone deacetylase inhibitors (HDACi) are used for some hematologic malignancies, and DNA methylation inhibitors are approved for treatment of myelodysplastic syndrome. HDACi are also being tested in psychiatric, neurologic and neurodegenerative diseases. The most known example is the short chain fatty acid

Valproate, which is used as a mood stabilizer and as an anti-epileptic drug. In nephrology, losartan, an ARB-blocker, may be a drug of interest as it mitigates DN features in diabetic mice and reduces histone mark H3K9/14Ac at key pathologic genes associated with diabetic glomerulopathy [200]. ARBs also have therapeutic effects on proteinuric CKD, however not via deacetylation activities but rather via modulation of DNA methylation [201]. In mice, the use of DNMT inhibitors has been studied in the context of uraemic toxin-induced CKD progression. The epigenetic silencing of the renoprotective *KL* gene by uraemic toxins, which is induced via elevated DNMT protein expression and subsequent hypermethylation, could be reversed by treatment with DNMT inhibitors [121]. Thus, targeting DNA methylation sites of specific genes may represent an effective strategy to modulate progression of CKD [202]. Use of these drugs alone or in combination with other epigenetic modulators, may be evaluated for improved treatment effects in CKD. Since observations from the use of DNMT inhibitors in models of neuroendocrine/psychiatric disorders have shown no adverse events, clinical trials in humans have been initiated [203]. The general concern regarding the targeting of epigenetic drugs to epigenetic modifiers, is the lack of gene locus specificity. This problem may be circumvented with the CRISPR/Cas9 technology. In a recent study, the epigenetic modifiers DNA methyltransferase DNMT3a or the Tet1 (part of DNA demethylation) was joined with catalytically inactive Cas9 into a fusion protein that was able to alter the methylation state - with functional effects - at specific DNA sequences [204]. As these experiments demonstrate that clinical epigenetic editing in specific loci in specific cell types may be possible, this would open up for exciting therapeutic possibilities.

Epigenetic drugs that delay premature ageing and prolong lifespan in CKD would be of great interest. Calorie restriction is robustly associated with extending health and longevity in several biological model systems [205]. The possibility to modulate these systems via calorie restriction mimetics includes modulation of pathways involving insulin/insulin growth factor-1, mammalian target of rapamycin (mTOR), and 5' adenosine monophosphate-activated protein kinase (AMPK), of which resveratrol, metformin, rapamycin and sirtuin modulators are the most extensively studied [206]. Sirtuins, which regulate diverse cellular functions via epigenetic modification of histones and other proteins, are considered guardians of the mammalian health span [207].The sirtuin family is conserved across taxa and displays NAD+-dependent deacetylase, deacylase, desuccinylase, demalonylase, deglutarylase and ADP-ribosyltransferase activities [208], thereby enabling a dynamic response to redox, circadian and metabolic changes [209]. Thus, in line with these activities, Sirtuin modulators have been

associated with beneficial effects in human pathologies including neurodegeneration and cancer [210].

**miRNA-based therapy in CKD**

A miRNA-based therapy that either restores or blocks miRNA expression and activity (miRmimics/pre-mirs or antisense RNA molecules, respectively) *in vivo* is very attractive, especially now that the first miRNA-targeting drug (miravirsen for the treatment of hepatitis C) has entered a phase II clinical trial [211]. During the last decade, most focus has been on the anti-miR therapeutics rather than the miR mimics, and several miRNA-targeting drugs entered clinical trial testing [212]. In CKD, animal studies have shown that the *in vivo* use of specific miRNA antagonists is an effective anti-fibrotic therapy [213-215]. However, some pitfalls have to be taken into account: first, the mutual regulation of miRNAs and target genes is challenging our understanding of the gene-regulatory role of miRNAs *in vivo* and has important implications for the use of these RNAs in therapeutic settings. Next, delivery and safety issues should be transparent.

MicroRNAs are also believed to play a role in the pathogenesis of acute kidney injury (AKI) induced by renal ischemia-reperfusion, which is a major kidney disease with no effective therapy available at present. Specifically, studies of cultured tubular cells have shown that the transcriptional factor hypoxia-inducible factor-1 (HIF-1), targeting genes involved in erythropoiesis and angiogenesis to increase oxygen delivery, induces miR-687 expression during hypoxia. Upregulation of miR-687, in turn, results in repression of the tumour suppressor phosphatase and tensin homolog (*PTEN*) gene, cell cycle activation and accelerated kidney repair [216]. Intriguingly, in vivo studies showed paradoxical results, as mice treated with anti-mir-687 were protected against kidney injury. One possible explanation for the discrepant outcomes between studies of in vitro and in vivo models may be the presence of other cells than tubular cells in vivo, as suggested by Nangaku M *et al* [217]. Apart from epigenetic regulation via miR-687, HIF-1 also targets histone lysine demethylases (a.k.a. Jumonji C lysine demethylases) under conditions of oxygen deprivation, thereby modifying histones and chromatin conformation [218]. Future studies will tell if the HIF-1/miR-687/PTEN signalling pathway is a candidate for the development of safe and effective therapeutic drugs to treat kidney damage.

**Life-style interventions as a way to interfere with epigenetic changes**

As epigenetic alterations of gene expression act as a dynamic response to changes in environmental conditions, e.g. inflammatory, redox and nutritional stress, lifestyle interventions, such as exercise and diet, have great potential to prevent or counteract detrimental changes of the epigenome associated with disease and ageing [133]. For instance, both DNA methylation patterns and histone modifications in human skeletal muscle responds to acute exercise [219, 220]. Moreover, acute exercise and exercise training result in changes in several circulating miRNAs in healthy volunteers, athletes and in CKD [110, 221, 222]. Differential expression of miR-210 (involved in adaptation to hypoxia) in response to an acute exercise bout in CKD patients who followed a formal training program, may give a clue towards understanding the mechanisms underlying the beneficial and rejuvenating effects of physical activity. Indeed, lifestyle intervention comprising physical activity is an effective way to reduce high CV risk in CKD patients [223].

## SUMMARY AND CONCLUSIONS

Epigenomic studies may contribute to a better identification of patients at increased risk for CKD and/or an increased disease burden by designing improved genetic-epigenetic risk profiles. Better understanding of the inter-individual variations in progression and outcome in CKD is a first step in bringing precision medicine into renal care, both in the preventive and therapeutic setting (e.g. lifestyle modifications, nutritional and pharmacological interventions). Epigenetic analyses also provide a platform enabling researchers to understand in-depth pathways involved in disease and could provide an explanation for divergent transcriptomic and proteomic data. Since epigenetics calibrate the genetic code this opens up for new therapeutic possibilities to reprogram our genome by modulating the gene transcription machineries to turn on or off genes that are identified as either protective or harmful. In the future, longitudinal studies are needed to assess the longevity and stability of epigenetic modifications in CKD. Given their close interrelation, the different elements of the epigenetic landscape should be studied as a whole and in context of genetic variation as well as variability between and within tissues in the uraemic milieu.

## ACKNOWLEDGEMENTS

Funding was provided by The Swedish Medical Research Council, Heart-Lung Foundation, Loo and Hans Osterman Foundation for Medical Research, Njurfonden, AFA Insurance and Karolinska Institutet Research Funds. A. Van Craenenbroeck was supported by an ERA-EDTA Short Term Fellowship.

## DECLARATIONS OF INTEREST

The authors have no conflict of interest to declare.

## AUTHOR CONTRIBUTION STATEMENT

All authors contributed to drafting, writing and editing the manuscript. All authors have read and approved the final version of the manuscript for publication.

## FIGURE LEGENDS

**Figure 1.** Patients with chronic kidney disease (CKD) seem to be subjected to an accelerated ageing, which put them at high risk for developing serious comorbidities such as vascular disease and premature death. The inter-individual variability is however large. This discrepancy is likely explained by many interacting risk factors, both in the endogenous and exogenous milieu, which may influence the uraemic phenotype via epigenomic modulations on gene expression as well as aberrant loss of imprinting, chromosomal instability and telomere attrition. Increased knowledge of the epigenetic drivers of the uraemic phenotype may be used to design genetic-epigenetic-based risk profiles, diagnostic tools and to develop personalized interventions and therapeutics for CKD patients.

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