New Biomarkers Meriting Evaluation for Use with BioSCIM

SUPPLEMENTARY MATERIAL for:

"**Monitoring Wastewater for Assessing Community Health: Sewage Chemical-Information Mining (SCIM)**"

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1. Introduction

The following Supplementary Material provides brief discussions of each of the major biomarkers selected in this assessment that merit more detailed examination for whether they might be suitable for use with BioSCIM. These select few markers certainly do not serve as an exhaustive list. The intention was instead to offer some examples that reflect the types of information required to assess whether biomarkers might make suitable candidates. These summaries are presented with the caveat that additional major limitations may have been overlooked in the review of the literature - limitations that could nullify the utility of these markers. Each of these biomarkers is summarized in Table 2 of the manuscript.

2. Desmosines

The desmosines comprise two non-natural, tetrafunctional amino acids, each derived from four lysine residues that are synthesized within the matrix of elastin. Found only in elastin, their cross-linking confers structural longevity while allowing an elastin network to stretch and recoil reversibly in all directions. Known as desmosine (DES, a 1,2,3,5-tetrasubstituted pyridinium amino acid) and isodesmosine (IDES, a 1,3,4,5-tetrasubstituted pyridinium amino acid), these are collectively called the "desmosines". Elastin is the protein that imparts the elasticity required for many connective tissues, especially pulmonary and cardiovascular systems.

Significantly, elastin is synthesized only during growth years, after which its continued synthesis is repressed. The desmosines are rather unusual in that they are available to the body only in a finite supply. They are therefore excreted in finite amounts over a lifetime - as a result of damage and resulting breakdown by elastase. As such, their excretion is negatively correlated with health. Damage to elastin (which occurs from a number of diseases) releases the desmosines - in both free form and also conjugated to other proteins. The desmosines are extensively excreted in urine and are very chemically stable; desmosines reach higher levels in urine as a result of regulatory control of blood levels, which is achieved by removal via kidney filtration.

The desmosines have been known since the 1960s, with hundreds of papers having been published regarding their potential use as biomarkers in tracking the progression of pulmonary diseases - especially chronic obstructive pulmonary disease (COPD) (Iadarola and Luisetti, 2013; Ongay et al., 2016; Viglio et al., 2017); note, however, that excreted levels can decline in advanced disease stages as elastin becomes depleted. Desmosines can also be released from malignant solid tumors (Starcher et al., 2013). Their accelerated excretion is also a natural consequence of the normal aging process, which often can become entangled with disease processes (the convolution of biomarker levels resulting from disease and from natural aging processes is a problem shared with many biomarkers).

Since COPD continues to grow as a public health epidemic, the ability to monitor desmosines using BioSCIM would hold considerable potential value as a measure for a specific disease process. DES has also not proved controllable by drug intervention, so this eliminates drug therapy as a confounder for excreted amounts. Dietary

elastin (as a potential extraneous source of desmosines) is uncommon in Western diets; dietary beef is reported to not affect urinary levels of desmosines (Laguna et al., 2013). As with most biomarkers, many unknowns need to be addressed before an assessment can be made for the utility of desmosines with BioSCIM. Two of the few reviews of desmosines as biomarkers were published by Iadarola and Luisetti (2013) and Viglio et al. (2017).

3. Bone Turnover Markers: DPD, NTX, and CTX

Various specific biomolecules are used in clinical medicine as bone turnover markers (BTMs) to monitor the continual, ongoing process of bone remodeling. Bone remodeling is critical for maintaining the structural integrity and health of bone (Guañabens et al., 2015). The value of these tests resides in tracking trends in the rate of change (to monitor disease progression) - especially to determine if net bone resorption (breakdown) is occurring. This is dictated by the balance between the activity of osteoblasts and osteoclasts; the former create new bone (e.g., mineralization), but only after the osteoclasts initiate resorption in appropriate areas of the bone. Net bone formation and integrity (e.g., density and strength) tend to occur only in the growth years. Net bone loss and integrity begins at around ages 25-30; gender differences can be profound. This natural process, however, can be disrupted by a variety of diseases as well as from prolonged use of certain drugs (glucocorticoids and anti-epileptics are but two examples). Excessive resorption can be a major concern (e.g., loss of bone mass and strength) and results in elevated BTMs. Noteworthy is that most BTMs are measured in blood and would clearly not be useful for BioSCIM applications.

With respect to BioSCIM, it is unclear if BTM measures from numerous pooled individuals would be meaningful, especially given that natural diurnal fluctuations in excreted levels from any given individual can vary considerably (e.g., 50% or more). This might obscure the interpretation of BTM data from BioSCIM even further. With this said, the primary urinary BTMs that might be of use fall into two classes - each measuring resorption acting on a different structural facet of bone-collagen. Both of these classes are integral to the physical structure and stability of type I collagen, which is the major constituent of the organic matrix of bone.

Type 1 collagen fibrils comprise triple helices of amino acid chains dominated by proline and glycine. The fibrils each terminate with non-helix carboxy and amino sequences. These two sequences represent the first class of potential BTMs - the "terminal telopeptide crosslink" sequences; a telopeptide comprises an amino acid sequence required for initial formation or conformation of a protein but which is later removed upon maturation of the protein. These urinary telopeptides are eventually released during bone resorption. The principal ones are NTX and CTX: "N-terminal telopeptide crosslinks" (amino-terminal collagen crosslinks) and "C-terminal telopeptide crosslinks" (carboxy-terminal collagen crosslinks), respectively; note that a number of isomers also exist but are not discussed here.

Within the collagen matrix, the telopeptide sequences are bound to the second major class of urinary BTMs - the collagen cross-linking pyridinolines [pyridinoline (PYD) and deoxypyridinoline (DPD, or D-PYR)], which are tri-

substituted amino acid derivatives of hydroxypyridinium. Each tri-functional pyridinoline links a terminal telopeptide sequence of one triple helix to the body of the triple helix of an adjoining fibril, thereby conferring physical stability to the eventual collagen macrofibre. This is a greatly simplified description of the structural role played by these constituents in type I collagen fibrils. The crosslinked structure is illustrated and discussed in Hlaing and Compston (2014).

During bone resorption, the likely major urinary markers that result from collagen degradation are DPD, NTX, and CTX. DPD, unlike PYD, occurs almost exclusively in bone and dentin and is therefore preferred as a marker for bone-related diseases (such as osteoporosis) (Hlaing and Compston, 2014). Note, however, that urinary PYD and DPD could also result from types of collagen other than type I. But bone represents the largest source. Potentially of significance is that urinary DPD levels may not be influenced by diet (Nishizawa et al., 2013), but it is unknown if BTMs in raw or digested foods might be released to sewage and therefore confound BioSCIM data.

4. Pteridines: Neopterin

Pterins are based on the bicyclic 4-keto-2-amino pteridine ring. Neopterin is a catabolic by-product of cyclic guanosine triphosphate - upstream in the tetrahydrobiopterin biosynthetic pathway. Its production results primarily from activation of the cell-mediated immune system (Ghisoni et al., 2016); in this sense it serves as a marker for a properly functioning immune system, which can be interpreted as a healthy response to a stress. Neopterin is produced in rather large quantities as a result of a wide spectrum of infectious diseases and some diseases caused by - or associated with - excessive inflammation (Burton and Ma, 2017; Geisler et al., 2015; Janmale et al., 2015; Taymur et al., 2015). As such, neopterin serves to integrate the collective stress from a large number of adverse conditions. In this sense it is similar to isoprostanes in reflecting collective exposures to numerous, diverse stressors involved with systemic oxidative damage. But since a compromised (impaired) immune system might not produce elevated levels of neopterin, it might be difficult to interpret on balance whether its production predominantly indicates health or stress. Neopterin is excreted both via urine and feces (Buchman, 2016).

A potential limitation for BioSCIM is that neopterin also seems to occur as a natural product in some foods, such as tomatoes, spinach, and beets (Martin-Tornero et al., 2016; Rodrigues da Silva et al., 2007). This could confound BioSCIM data if raw or cooked foods entered sewers, especially since boiling and oxidation may lead to complex patterns of pteridine-interconversions and degradation (Van Daele et al., 2016). Daily variance in intra-individual excretion is also increased as a result of endogenous factors affecting its synthesis (Burton et al., 2016). Although neopterin is relatively stable (Heistermann and Higham, 2015), it is susceptible to UV photolysis (Behringer et al., 2017; Laich et al., 2002). This could cause losses in sewage open to the air, as well as in collected monitoring samples not shielded from the sun.

5. DAMPS: *N*-formyl-Met-Leu-Phe (fMLP)

fMLP is a member of the class of "alarmin" or "danger signal" molecules called "mitochondrial-derived damage-associated molecular patterns" (mtDAMPs) or "chemotactic factors". These chemotactic peptides are released from mitochondria. Once they become available extracellularly, such as from localized necrosis or serious systemic inflammation, they attract neutrophils (via surface receptors) and stimulate oxidative bursts. An integral part of the innate immune system, this simple tri-peptide (*N*-formylmethionine-leucylphenylalanine) serves as a mimic for the same peptide sequence (as well as for other bacterial oligopeptides) released by pathogenic bacteria (Pugin, 2012). Unfortunately, the bacterial source might serve to confound the human origin of fMLP as a suitable BioSCIM marker. While fMLP is clearly excreted in urine, it is also known to occur in agricultural and house dusts (Castranova et al., 1996). It is currently unknown whether fMLP also occurs at ambient levels in sewage after release from dusts or from bacterial lysis. Experiments would first have to be designed to determine if there were evidence for exogenous levels of fMLP that exceeded those expected just from urine/feces.

6. Polyamines: Diacetylspermine (DAS)

The polyamines (PAs) comprise a class of important biochemicals with rather complex metabolic pathways (Miller-Fleming et al., 2015). Traditionally, the PAs of usual focus in physiology are the aliphatic polyamines: putrescine (a diamine), spermidine (a triamine), and spermine (a tetraamine). These three constitute a largely reversible metabolic cycle (downstream of the arginine-ornithine pathway) that serves essential regulatory and control functions for all mammalian cells (primarily via PA-RNA complexes). But with regard to utility with BioSCIM, it is some of the PA metabolites that become ejected from this cycle that hold the most potential as useful biomarkers for a variety of diseases. The *N*-acetyl catabolic derivatives (generated via spermidine/spermine N^1-acetyltransferase - SSAT) have shown the most promise, as they are extensively excreted in urine. The monoacetyl derivatives, however, are also extremely abundant. Acetylputrescine along with N^1- and N^8-acetylspermidine account for over 90% of the polyamines excreted in human urine (Kawakita et al., 2015). The abundance of the non-acetyl and mono-acetyl PAs in urine negate their utility in discerning perturbations in their excreted levels.

The PA cycle is closely controlled to maintain homoeostatic levels of the principal circulating polyamines. But during active tumor growth, homeostasis is perturbed to the extent that concentrations of many of the metabolites become elevated. Of particular promise as biomarkers are the diacetyl polyamines, which are produced at much lower levels. N^1,N^{12}-diacetylspermine (DiAcSpm or DAS) in particular comprises only about 0.5% of the total urinary PAs. But the lower excreted levels of DAS have proven more useful in revealing changes. DAS also shows remarkably little intra-individual or inter-individual daily variation among healthy individuals (Hiramatsu et al., 2014); this fact may prove important with respect to the potential value of DAS as a urine-hydration normalization marker (an alternative to creatinine). But most notably, DAS levels become elevated with the onset and progression of a number of different cancers (Kawakita and Hiramatsu, 2006; Nakayama et al., 2012; Park and Igarashi, 2013;

Pegg and Casero, 2011; Takahashi et al., 2015), pointing to their potential value as BioSCIM markers of disease, especially cancers.

Polyamines have become a primary focus in the development of biomarkers for cancers, especially since SSAT becomes upregulated, leading to disruption of PA recycle and enhanced production and accumulation of the di-*N*-acetyl derivatives (Kawakita and Hiramatsu, 2006; Kawakita et al., 2011). The published clinical literature on PAs is quite extensive but most is only peripherally relevant for BioSCIM. One of the major concerns with respect to BioSCIM is the extent to which DAS might occur in the diet [e.g., a wide array of foods, such as cheese and citrus, are rich in the non-acetylated polyamines (Kalač, 2014)], as this would confound the utility of DAS for BioSCIM; but acetylated PAs (especially diacetyl derivatives) seem to occur in plants at very low levels (mono-acetylspermine, for example, tends to be back-converted to spermidine) (Ahou et al., 2014). Bacterial biofilms in the gut also metabolize polyamines (Johnson et al., 2015). Finally, the polyamines are very chemically stable with respect to heat and pH, indicating that they may persist in sewage.

Also noteworthy is the role played by three of the polyamines in generating reactive aldehydes, which in turn lead to the generation of cytotoxic acrolein. Acrolein can then react with lysine residues, generating another potential biomarker - the advanced lipoxidation end-product **FDP-lysine** [N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine], which is a specific measure of protein-conjugated acrolein (Park and Igarashi, 2013; Tomitori et al., 2005) and also a marker of cumulative oxidative stress (analogous to the archetype BioSCIM biomarker class - the isoprostanes).

7. p75 Neurotrophin Receptor [Extracellular Domain]

Neurotrophic factors, which range from peptides to small proteins, mediate the development and survival of neurons. Nerve growth factor (NGF) is a well-known example (Skaper, 2017). One of two receptor types for NGF is neurotrophin receptor p75 (p75NTR), also called low-affinity nerve growth factor receptor (LNGFR). During embryonic development, p75NTR is up-regulated. It is then down-regulated to consistent, low levels for the remainder of life. It is re-expressed, however, upon neuronal injury or the onset of certain neurological diseases (Shepheard et al., 2014). A specific portion of the transmembrane p75 neurotrophin receptor is referred to as the extracellular domain (ECD, or ectodomain) and abbreviated $p75^{ECD}$; in earlier literature, this ectodomain was often called the truncated nerve growth factor receptor (tNGFR or NGFRt).

During neuronal development, injury, or disease, $p75^{ECD}$ is enzymatically cleaved from the transmembrane receptor via alpha-secretase. Subsequent shedding of the water-soluble $p75^{ECD}$ results in its extensive urinary excretion. Urinary levels faithfully track normal neuronal development and maturation (DiStefano et al., 1991) as well as the onset and progression of neurological disease - notably amyotrophic lateral sclerosis (ALS) (Shepheard et al., 2017). In the latter study, urinary levels of $p75^{ECD}$ from ALS patients are reported to be up to 5-fold higher than controls (but generally less than 2-fold). Excreted levels track disease progression, but overlap is observed in excreted levels

across groups. Just the same, the assay was deemed robust, as excretion was unaffected by time of day or gender, and no losses were observed in urine samples levels stored at different temperatures.

Overall, $p75^{ECD}$ has the hallmarks of a potential alternative to creatinine for healthy individuals. Its major strength resides in its very constant daily excretion rates, with much less inter-individual variance compared with most other biomarkers. Since it is unknown how many diseases other than ALS substantially increase its excreted levels, it is unclear whether its levels in sewage would be sufficiently sensitive to reveal change (especially since these neuronal diseases usually occur at very low rates within a population). While this might reduce its value for BioSCIM as a disease biomarker, these same characteristics would make $p75^{ECD}$ an intriguing alternative to creatinine. But complications arise from the fact that excreted levels are significantly elevated in the last trimester of pregnancy and during the first month or so of life (DiStefano et al., 1991). This would require that the age distributions and rates of pregnancy within populations would need to be comparable. Another potentially limiting factor might be that levels in sewage are close to, or below, the limits of analytical detection, as the reported excreted levels in urine seem to be in the range of nanograms per mg of creatinine. After dilution of urine by 3 or more orders of magnitude in sewage, the ultimate levels could pose challenges for detection. Time-trend variations might then not be distinguishable from the signal added by the incidence of disease.

8. Urinary proteins associated with kidney dysfunction or injury

Despite the reality that thousands of endogenous proteins - many of which are excreted in urine [e.g., see: Adachi et al. (2006)] - might serve as useful biomarkers, years of research are required to identify and validate those of clinical use. A useful way to reduce the numbers of candidates deserving further examination is to focus on those involved with kidney diseases, as they are most likely to be extensively excreted in urine - and often at high levels (e.g., see: Gao, 2015). Only two examples of potential candidates are briefly presented here: vitamin D-binding protein (VDBP) and monocyte chemoattractant protein-1 (MCP-1). The discussion is limited because there is less known about these that would be relevant to BioSCIM. With respect to molecular size, note that the median size for human proteins is roughly 40 kDa. With respect to BioSCIM, a major unknown with nearly all proteinaceous markers is their stability or persistence in sewage (as opposed to urine).

8.1. Vitamin D-binding protein (VDBP)

VDBP is a 58-kDa glycoprotein, also known as Gc-globulin. VDBP binds with vitamin D metabolites and serves to transport and to protect/recycle them in circulation; it also works in concert with gelsolin (discussed below) to scavenge actin. VDBP is present at very high concentrations in the serum of healthy individuals: around 0.4 mg/mL (Bikle et al., 1986). This contrasts starkly with the extremely low urinary levels from healthy individuals (Chaykovska et al., 2016; Mirković et al., 2013) – a result of highly efficient renal reabsorption. The levels of VDBP excreted during the development and especially manifestation of a variety of kidney diseases or injury, however, can become greatly elevated, sometimes by several orders of magnitude (Mirković et al., 2013; Shoukry et al., 2015;

Tian et al., 2014). This is significant in that the relative levels between healthy and diseased states is much larger than for most biomarkers. This would greatly enhance the odds of successful detection in sewage.

8.2. Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) is a 13-kDa cytokine, also known as CC-chemokine ligand 2 (CCL2) or small inducible cytokine A2. It is renowned as the most potent chemotactic factor for monocytes. It becomes over-expressed with renal disease such as diabetic nephropathy. Compared with VDBP, the excreted levels of MCP-1 between healthy and diseased states do not change as much, but they still change by over an order of magnitude (Mansour et al., 2017a; Shoukry et al., 2015). Increased urinary levels of MCP-1 can be confounded by acute kidney damage, simply as a normal physiological response resulting, for example, from strenuous, sustained exercise (Mansour et al., 2017b).

9. Gelsolin

Gelsolin was briefly mentioned in the section "The Acute-Phase Response (APR)." Gelsolin is a protective agent, informally known as one of the more potent "actin scavenger" proteins; vitamin D binding protein (discussed above) is another (Shen et al., 2012). With a ubiquitous intracellular and extracelluar systemic distribution, its rather high plasma levels in healthy individuals range from 200 to 300 mg/L. It binds not just actin, but also a spectrum of other bioactive, and often deleterious, biochemicals. It plays a key role in regulating the disassembly of actin filament that has been exposed or released from cellular injury and which can reach toxic levels. By severing the free filaments, it can promote the conservation and remodeling of actin filament. Actin's cross-linked gel structure is effectively solubilized by *gel-sol-in*, hence the name. Gelsolin levels can therefore become depleted after cellular injury. And its depletion is prognostic of adverse outcomes; it therefore acts like a negative acute-phase protein. Overall, its elevated levels serve as a marker for health (Kustán et al., 2017; Nag et al., 2013; Park et al., 2016; Peddada et al., 2012).

Gelsolin itself is cleaved by caspase-3, releasing the terminal (or truncated) fragment, t-gelsolin (or tGelsolin); this fragment can also sever actin, but not in a regulated manner. Because of gelsolin's larger size (82 kDa) compared with its proteolytic fragment t-gelsolin (43 kDa), the fragment more easily escapes glomerular filtration from the blood than gelsolin itself and is therefore more easily excreted in the urine. This difference in sizes explains why most data on urinary levels involve gelsolin fragments such as t-gelsolin (Kothakota et al., 1997; Li et al., 2012; Sakurai and Utsumi, 2006). There is little data reported on urinary levels of parent gelsolin. High plasma levels of gelsolin can become translated into low gelsolin fragment levels in the urine, and vice-versa. As with other negative APPs, gelsolin can also be up-regulated in some disease states, so its plasma levels may not be easily interpreted as reflecting health or stress. It is unclear whether these potential problems might serve to confound the interpretation of urinary data.

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