Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype

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Timely assessment of the aggregate health of small-area human populations is essential for guiding the optimal investment of resources needed for preventing, avoiding, controlling, or mitigating exposure risks. Seeking those interventions yielding the greatest benefit with respect to allocation of resources is essential for making progress toward community sustainability, promoting social justice, and maintaining or improving health and well-being. More efficient approaches are needed for revealing cause-effect linkages between environmental stressors and human health and for measuring overall aggregate health of small-area populations. A new concept is presented - community health assessment via Sewage Chemical Information Mining (SCIM) - for quickly gauging overall, aggregate health status or trends for entire small-area populations. The approach - BioSCIM - would monitor raw sewage for specific biomarkers broadly associated with human disease, stress, or health. A wealth of untapped chemical information resides in raw sewage, a portion comprising human biomarkers of exposure and effects. BioSCIM holds potential for capitalizing on the presence of biomarkers in sewage for accomplishing any number of objectives. One of the many potential applications of BioSCIM could use various biomarkers of stress resulting from the collective excretion from all individuals in a local population. A prototype example is presented using a class of biomarkers that measures collective, systemic oxidative stress - the isoprostanes (prostaglandin-like free-radical catalyzed oxidation products from certain polyunsaturated fatty acids). Sampling and analysis of raw sewage holds great potential for quickly determining aggregate biomarker levels for entire communities. Presented are the basic principles of BioSCIM, together with its anticipated limitations, challenges, and potential applications in assessing community-wide health. Community health assessment via BioSCIM could allow rapid assessments and intercomparisons of health status among distinct populations, revealing hidden or emerging trends or disparities and aiding in evaluating correlations (or hypotheses) between stressor exposures and disease.

Keywords: exposure assessment, sewage epidemiology, public health, oxidative stress, isoprostanes, biomarkers

Abbreviations:
AA: arachidonic acid
BioSCIM: sewage chemical-information mining targeted at biomarkers
BMI: body mass index
BOSS: Biomarkers of Oxidative Stress Study (of the National Institute of Environmental Health Sciences)
COX: cyclooxygenase
DHA: docosahexaenoic acid

EPA: eicosapentaenoic acid
FEUDS: Forensic Epidemiology Using Drugs in Sewage
HBM: human biomonitoring
HPLC: high-performance liquid chromatography
IsoP(s): isoprostane(s)
MS: mass spectrometry
NSAIDs: non-steroidal anti-inflammatory drugs
PUFAs: polyunsaturated fatty acids
SCIM: sewage chemical-information mining
STPs: sewage treatment plants

Contents

1. Introduction

An efficient, timely, and holistic approach for revealing cause-effect linkages between environmental stressors and the health of human populations persists as an unmet need. Presented here is a new concept that targets biomarkers of endogenous human metabolism that serve as measures of actual exposure and effects from a wide spectrum of stressors extending beyond the traditional focus limited strictly to chemicals. These biomarkers are normally hidden in a uniquely rich source of chemical information that has been largely ignored - - raw (untreated) sewage. The concept - - termed *BioSCIM* (sewage chemical information mining targeted at biomarkers) - - could make possible the real-time collection of exposure/effects data that reflects the overall, averaged health of entire small-area communities.

Measures of community-wide health are rare. The only means of assessment is currently limited to morbidity and mortality data and conventional demographic data; included in morbidity data are specific sources such as poisonings and epidemiological studies (e.g., human biomonitoring). Morbidity data is not necessarily representative of an entire population (because of the incompleteness in reporting). It is also not possible to correlate any of these data with a defined population (or with stressors) in real-time.

Resource- and time-intensive epidemiological studies have been the primary route by which specific cause-effect linkages can sometimes be revealed. Indirect approaches, such as the CDC's National Biomonitoring Program (CDC, 2010), establish body burdens for a narrow spectrum of pre-selected xenobiotics in various body tissues and fluids (two examples being the analysis of biopsies for PCBs or halogenated pesticides, or urine for phthalates). Biomonitoring for chemical stressors, however, only provides the means for inferring the potential for exposure. Biomonitoring is unable to address questions of linkage with human health effects. The body burden of a chemical may simply be coincidental and of no biological consequence. Although development of new approaches for assessing human exposure is an ongoing task, most rely on advanced technology and are limited by the laborious requirements and hurdles imposed by the

need to test individuals (e.g., Borrell, 2011). They are simply not suitable for quickly assessing exposure across entire populations.

A more direct approach for discovering potential linkages between environmental exposure and biological effects is the measurement of endogenous biomarkers. In the context of human health, biomarkers include physiological or behavioral responses, or endogenous biochemicals produced in response to: normal biological processes (known as biomarkers of health), disease processes (as induced by exposure to a biological, physical, radiological, psychological, or chemical stressor), or pharmacological intervention (Biomarkers Definitions Working Group, 2001).

Conventionally, retrospective assessments (e.g., epidemiology; biomonitoring) or prospective assessments (e.g., predictions based on toxicology or in silico methods) have served as guides. But neither of these approaches provides the ability to gauge collective, population-wide health status or trends, nor can they provide measurement in real-time. Consequently, they are not useful for supporting the use of short-term experiments (testing "what-if" scenarios) or trial interventions that are essential for evaluating the outcomes from actions intended to improve or maintain population-wide health. Nor are they suitable for performing comparisons among small-area populations - - an especially important need for revealing disparities regarding exposure or health between communities (e.g., environmental justice). In this sense, the BioSCIM concept presented here can be viewed as a convenient means to facilitate or capitalize on conducting so-called "natural experiments." These quasi-experiments are strictly observational, with non-randomized designs that cannot be influenced by the researcher and which occur "as is" in the course of ongoing events. Natural experiments are one of the only approaches for evaluating the health outcomes from community-wide interventions - - whether targeted or not at influencing public health. In this sense, they could play important roles in public health research, especially for study of interventions intended to reduce health inequalities (Petticrew et al., 2005).

Adding further complexity is the need to determine the comparative impacts on health resulting from disparate classes or types of stressors among the vast spectrum of possibilities (both anthropogenic and naturally occurring) to which humans are exposed. Currently not possible are

meaningful assessments of the relative importance of exposure to the continuum of chemical toxicants (singly or in combination; naturally occurring or anthropogenic), especially at the trace levels normally occurring in the ambient environment. Even more insurmountable obstacles are faced in assessing the relative importance among all stressors - - prioritizing across chemical, biological, physical, radiological, and psychological (e.g., social or emotional) stressors. Further discussion of the background and rationale underlying the proposed BioSCIM concept is presented in the Supplementary materials.

The proposed BioSCIM concept could serve as a key indicator in the "Three Pillars" approach for sustainability, a rapidly evolving paradigm that must account not just for environmental factors, but also social and economic factors in environmental decision making (NRC, 2011). Since specific aspects of human health are impacted by a confluence of interlinked environmental, social, and economic factors, BioSCIM indicators could serve as integrative measures for sustainability. The application of BioSCIM to monitoring collective, population-wide health holds potential for more effectively guiding the development of sustainable communities and in reducing inter-community disparities by facilitating the most effective interventions or corrective actions in greatly reduced time frames. This ability is important in the Environmental Protection Agency's vision, especially for measuring health disparities in vulnerable groups and for demonstrating outcomes from numerous decision, compliance, and enforcement activities (USEPA, 2010). A collective measure of stress would hold the potential for making it possible for the first time to facilitate pinpointing which individual stressors play the most important roles in community-wide disease - - whether the stress is chemical or non-chemical.

2. Biomarkers in sewage - - *BioSCIM*

Proposed here is a concept for performing "remote" community-wide biomonitoring - - a means for monitoring the status or trends in the collective health of small-area populations; the nature of small-area populations and how their size can be measured has been presented (Daughton, 2012). The approach is highly innovative but also conceptually simple - - measuring biomarkers

collectively excreted by a community into sewage. If successful, it would provide for the first time the ability to derive a measure of population-wide health status in a manner that has potential to be straightforward, fast, inexpensive, widely applicable, amenable to automation, and scalable (applicable to a broad spectrum of population sizes - - ranging from local neighborhoods to cities). Status or changes in community-wide health could be quantified and then be used to seek correlations with community-wide risk factors. Intra-community trends could be established, and comparisons made across communities. A new paradigm could emerge for justifying the prioritization of exposure risks and for determining the optimal use of resources for control or minimization of exposure risks.

The potential advantages of the proposed approach include: (i) acquisition of data on exposure/effects-based biomarker levels representative of entire populations in near real-time, (ii) eliminating the need to obtain samples from individuals (a form of "remote" monitoring), (iii) sufficient time-series community-wide data could be obtained for establishing status and trends, thereby making intra- and inter-community comparisons possible, and (iv) the impacts of risk-reduction actions could be more readily evaluated.

3. Generalized approach to developing a BioSCIM application

Development of a BioSCIM application begins with determining the most promising biomarker to first target in raw sewage. The complete criteria are outlined in Table 1 and summarized in a flow chart (Fig. 1). The major attributes include whether the biomarker is: (i) produced exclusively by disease or stress (i.e., not introduced by unrelated, exogenous mechanisms), (ii) excreted in sufficient quantities (to allow detection in sewage), (iii) sufficiently stable in sewage (not degraded, such as by microbial activity), (iv) amenable to cost-effective analysis in sewage while meeting whatever analytical figures of merit are required, and (v) excreted at levels in stressed/diseased states sufficiently elevated to discern significant differences compared with the narrow baseline range of "normal" basal states (see section 4: Reference intervals - - "normal" versus stressed).

An appropriate monitoring method would include standardized protocols for all steps beginning with sampling the raw sewage and concluding with data reporting (see flow chart, Fig. 1). Because of the anticipated natural variance in biomarker basal (baseline) excretion (a function of both intra- and inter-individual natural variation in excretion), coupled with the many irregularities associated with sewage flow, it would be critical to minimize the analytical variance to ensure that the signals are not obscured from any excursions in biomarker levels resulting from perturbations due to critical exposure events. A final hurdle would be the need to ensure that inter-laboratory variance is minimized - - to eventually enable meaningful intercomparisons between sewage treatment plants (STPs).

Importantly, the absolute accuracy of the analytical method would not be critical - - as long as the same standardized method is used in all BioSCIM studies; analytical bias would only need to be repeatable. But precision would be essential. The absolute concentration of a biomarker in sewage has little meaning in terms of clinical or epidemiological significance - - unless these levels could eventually be used to reconstruct average systemic levels within the individual - - in a manner analogous to the use of FEUDS (Forensic Epidemiology Using Drugs in Sewage) for reconstructing community-wide usage of an illicit drug based upon the drug's concentration in sewage (Daughton, 2011). In contrast, the reproducibility (and repeatable bias) of the method is critical for distinguishing statistically significant changes in excreted biomarker levels.

The two major uses for BioSCIM both involve the inter-comparability of per capita-normalized biomarker levels. These two uses would entail intra- and inter-STP relative comparisons. Relative values would rely on time-series data having sufficient precision.

The value of relative levels is illustrated with an emerging approach being implemented in a testing program for drug abuse in athletes. The "Athlete Biological Passport" uses drug monitoring designed only to detect changes (e.g., outliers) relative to the individual athlete's established personal profile of banned substances (those that are also endogenous substances, such as certain anabolic steroids) (Sottas et al., 2010). A major advantage in acquiring data for determining relative changes is that the method of analysis need only be precise - - yielding reproducible values. Absolute accuracy is not important.

Intercomparisons would be used for establishing current status and answering questions regarding trends (relative changes over time): Has a local per capita biomarker level changed over time? What percentage increase or decrease in per capita level has a community experienced over time? Is a local level trending upward or downward? How do the per capita levels or rates of change compare between local communities?

A relative measure could reveal those communities with the highest or lowest collective biomarker levels, or largest relative rate of increase, and thereby guide subsequent studies to locate the risk factors. A change in biomarker level could be viewed as analogous to a vehicle's "check engine" fault light for a community at large - - as an alert to a possible increase in existing stress or emergence of new stress.

Significant differences in inter-community comparisons could be used in conjunction with data-rich geographic information systems and demographic data to test for correlations with unlimited types of geospatial variables - - some obvious examples being noise (e.g., communities near airports, freeway interchanges, inner cities, green spaces), population density, proximity to toxic chemicals (e.g., hazardous waste sites) or other community disamenities (e.g., landfills), pesticide exposure (agricultural areas), radiation (high-voltage lines), population age structure, respirable particulates (freeway traffic or regional air quality), and per capita income. In this sense, BioSCIM would serve as a relatively quick screening tool that could then be used to justify and guide more in-depth follow-up examination of community-wide health as correlated with the presence or absence of various exposure sources.

4. Reference intervals - - "normal" versus stressed

Even if per capita basal levels of a biomarker for a particular STP exhibited a sufficiently narrow range of variability at any point in time - - making possible the detection of statistically significant trends - - a major question is whether it would be possible to assign the community an

absolute measure of "health" or "stress". To answer the question of whether a local per capita biomarker level is "abnormal" is considerably more difficult.

Fundamental questions surround what is even meant by "normal" (or "healthy") versus "diseased" - - or "non-diseased" versus "non-healthy". For the purposes of BioSCIM, it would require a comprehensive series of initial monitoring studies across carefully selected STPs - - those serving populations with well-documented overall health. These studies would be needed to establish "normal" ranges (baseline physiological levels for a population deemed to be "healthy") for the targeted biomarker. Data from individual sewage treatment plants (STPs) would serve instead of data from individual human subjects. Entire communities would be viewed as individual patients (Daughton, 2012).

These studies would be analogous to the clinical studies required for establishing the widely used *reference intervals* or *reference values* for blood or urinary levels of various markers - - a concept introduced in 1969 by Gräsbeck (2004). A unified approach for conceptualizing, defining, and developing reference values was begun in 1970 and summarized in a series of papers beginning in 1987 (Solberg, 1987). Despite the widely accepted and routine use of reference intervals in clinical chemistry for a wide spectrum of biochemical parameters, it is an extremely complex and controversial concept. It is one fraught with numerous challenges and far exceeds the scope of this paper. Comprehensive examination of the complexities, limitations, and pitfalls surrounding the development of reference intervals for laboratory medicine, which necessarily must accommodate natural biological variability, is available from a number of authors, including Apostoli et al. (1998), Gräsbeck (2004), Hyltoft-Petersen, and Henny and Petersen (2004).

The idea of examining an entire local community as if it were an individual patient poses many additional challenges than already encountered in establishing clinical reference values for inter-individual comparison. At the least, however, it should be possible for a local community to serve as its own reference (analogous to the clinical practice of "delta checking" based on intra-individual time-series reference values, or the Athlete Biological Passport mentioned above) if the contribution to intra-community variance (i.e., combined inter- and intra-individual variation

for "healthy" individuals - - due to both analytical variance and natural daily variation) - - is sufficiently small. A reference range for BioSCIM would at least only need the power to differentiate between a positive or negative relative change in status.

Regardless of whether it is possible to establish reference values (and intervals) for BioSCIM analytes, it would be possible to establish ranges based on (for example) the upper quintile (or lower quintile depending on whether the desired relative level of the selected biomarker should be high or low) of community-wide values. Values from a given STP could then be expressed relative to the extreme range, which could be termed the "target range" or some such - - a range that a community might strive to reach by implementing appropriate measures to improve health or avoid stress.

5. Sewage chemical information mining - - *SCIM*

Sewage can be viewed as "chemical litter" emanating from human behavior, actions, and activities. It has long been regarded as economically burdensome waste - - only recently viewed as a potential source of nutrients for agriculture or for reclaiming energy (e.g., Heidrich et al., 2010).

BioSCIM makes use of the wealth of chemical information present in sewage and relevant to health and disease. This rich but highly complex reservoir of potential biomarkers has never been mined. It resides in raw sewage in the form of unique signatures from countless chemicals excreted from the body as a result of myriad endogenous biochemical processes. In raw sewage, these substances represent the combined metabolic processes from all individuals composing a small-area population serviced by a discrete STP at any point in time. Applications of SCIM essentially serve as a form of community-wide, *en mass* urinalysis - - where single samples (collected over sufficient time) are necessarily representative of the entire population. The potential application of SCIM is flexible in terms of selecting well-defined small-area populations. The physical layout of sewer conveyance lines enables the collection of samples from different spatial locations, representing different segments of a population - - spanning

discrete hierarchical subgroups ranging from portions of a city, community, neighborhood, or particular street.

One way to apply BioSCIM in quantitative assessments necessitates knowing the size of the real-time population contributing to the sewage. This allows calculation of average per capita contributions (or loadings) of a biomarker. There are many limitations and complexities surrounding the concept of a small-area population. Their ramifications with respect to SCIM are discussed in Daughton (2012), as an application termed ASAP-SCIM (analysis of small-area populations by sewage chemical-information mining), which presents a new concept for estimating small-area population size by use of biomarkers whose constant levels of excretion reflect per capita contributions. An alternative approach for BioSCIM would obviate the need to directly know the population size. This could be done by monitoring for an orthogonal biomarker (such as a biomarker of health rather than stress) whose level the target biomarker level could be normalized against. This would create a dimensionless or normalized index (discussed in section 11*:* Differential Stress Index: Increasing the sensitivity of BioSCIM by also measuring biomarkers of health to derive a normalized index of stress or homeostasis).

The BioSCIM approach could also be viewed as a form of remotely sensed, collective HBM, where no active involvement or participation from any individual is needed. Although BioSCIM's economy of scale cannot generate data relevant to the individual, it generates time-series data and would facilitate quick inter-community comparisons. BioSCIM holds the potential for minimizing or eliminating the issues and concerns normally surrounding subject recruitment, retention and participation of recruits ("research fatigue"), informed consent, and confidentiality - - all of which are problematic for HBM studies and often even for epidemiology (Bauer, 2008). The non-invasive nature of BioSCIM would negate the need for recruiting and interacting with individual subjects and thereby obviate any requirement for Institutional Review Board approvals. This could greatly accelerate the pace of studies.

The NRC (2009) has noted the need for simpler screening-level tools for assessing cumulative risk - - especially at the community-wide scale. BioSCIM would serve as a first attempt at providing such a tool based on a biomarker of effect. As recommended by the NRC, the new

concept might also facilitate the conduct of risk assessments by community stakeholders, thereby empowering more involvement in evaluation, design, and management of local remedial or control activities. Access to near real-time data could facilitate the testing of what-if scenarios - - one example of many being encouraging change in community-wide behaviors and evaluating possible subsequent changes in BioSCIM biomarker levels. BioSCIM could create the opportunity to view communities from a completely new perspective - - where an entire community serves as the patient (Daughton and Ruhoy, 2011).

In addition to developing the framework for the BioSCIM remote biomonitoring concept, initial proof of principle is currently being planned by development of an analytical methodology for measurement of an effects-based biomarker in sewage. This may mark the first time that an effects biomarker has been targeted in the monitoring of sewage. Theoretically, comprehensive arrays of biomarkers suitable for monitoring in sewage could eventually be developed. Chemical information can be derived from the full spectrum of chemicals excreted by humans (including those in urine, feces, and sweat). These markers could range from general to specific in terms of the types and levels of biological effects they indicate.

5.1. Evolution of the SCIM concept: from FEUDS and ASAP-SCIM to BioSCIM

The BioSCIM concept derives from prior work directed at the extraction of chemical information from sewage. The proposed BioSCIM concept represents a new application of a general approach called sewage chemical-information mining - - SCIM (Daughton, 2012) - - specifically targeted at select biomarkers. The real-time monitoring of sewage for biomarkers as measures of community-wide health and disease was first proposed by Daughton (2011) and shortly thereafter by Thomas and Reid (2011); it has also been mentioned by Fanelli (2011). The work reported here resulted from a U.S. Environmental Protection Agency (EPA) 2010 research proposal on SCIM funded by EPA's ORD Pathfinder Innovation Projects (PIP) internal grants program (Daughton, 2010).

The first application of SCIM for extracting information from community-wide contribution of chemicals to sewage was introduced in 2001 (Daughton, 2001) and was first applied to real-world use in 2005 (Zuccato et al., 2008). This original concept (sometimes loosely referred to as "sewer epidemiology" or "sewage epidemiology") involved analysis of domestic sewage for illicit drugs (or their metabolites) and using pharmacokinetics and sewage flow rates to back-calculate aggregate and per-capita drug usage for an entire community served by the STP. This application represented the first time that aggregate dose-reconstruction could be accomplished at such a large scale. This application of SCIM has since been widely explored internationally. It was later termed Forensic Epidemiology Using Drugs in Sewage (FEUDS); for a review see Daughton (2011).

A second application of SCIM was introduced in 2011 and termed ASAP-SCIM: Analysis of Small-area Populations by Sewage Chemical-Information Mining (Daughton, 2012). This approach was proposed as a means of providing near-real-time estimates of small-area (local) populations by measuring select biomarkers excreted into sewage. ASAP-SCIM may prove useful in the implementation of BioSCIM. BioSCIM represents the third methodology based on SCIM and strives to monitor for biomarkers in sewage as a gauge of community-wide health. It is worth noting that most of the complexities associated with the actual implementation of FEUDS and ASAP-SCIM also apply to BioSCIM. So understanding the first two applications helps with understanding the limitations and challenges faced by BioSCIM.

Important to appreciate is the complexity surrounding the estimation of small-area population size in interpreting population-wide measures of health. When the small-area population (or change in population) is not accurately known, then the significance of absolute changes in health measures (mortality being one of the simplest examples) cannot be evaluated (Daughton, 2012). Any change in incidence can result from changes in any or all of three factors (Thunhurst, 2009): (i) absolute population (constant rate of incidence in a larger population results in higher occurrence rate), (ii) composition of constant population (changes in disaggregation, resulting in higher percentage of vulnerable individuals - - an example being an inverted age structure), or (iii) response of the overall population (e.g., changes in stressor levels). A methodology for easily resolving these factors is currently not possible.

A noteworthy aspect of the proposed BioSCIM approach is that it could theoretically also be extended for use in ecological assessments, especially given the emerging field of "oxidative stress ecology" (Costantini et al., 2010). It might prove particularly applicable, for example, in determining the health status of local aquatic environments - - by measuring select biomarkers excreted by aquatic organisms in their native environment; biomarkers of oxidative stress (already used in assessing individual organisms) constitute one example that may prove useful (Isaksson, 2010; Lushchak, 2011; McGraw et al., 2010; Metcalfe and Alonso-Alvarez, 2010).

6. Biomarkers.

Perhaps some of the most important information contained in sewage is represented by the countless excreted substances serving as biomarkers - - substances having potential as measures of collective community-wide disease, stress, or health. Biomarkers comprise three major categories in terms of what they can indicate (Hagger et al., 2006): exposure (e.g., as measured by dose), effects (e.g., diagnostic indicators, especially toxicity or other biological endpoint such as pathogenicity, or alteration of structure/function), and susceptibility or vulnerability (e.g., prognostic indicators, such as genetic polymorphisms); a summary of the requirements for biomarkers is presented in Breusing and Grune (2010). Biomarkers can therefore serve as diagnostic or prognostic measures of exposure, stress, vulnerability to disease, emerging disease, overt disease, or health. Biomarkers include endogenous biochemicals produced in response to stress or indicative of health. They also include adducts of xenobiotics or endogenous chemicals. And of course, they include metabolites of detoxication or intoxication processes resulting from xenobiotic exposure. Analysis of sewage for biomarkers, as a collective measure of population-wide health status or stress, has never been reported. Such an application would be aligned with the field of "molecular epidemiology" (Bonassi and Au, 2002), especially since a carefully selected biomarker can serve to integrate a diverse array of exposures - - regardless of type, source, or route.

Biomarkers are often used as proxy or surrogate measures of the actual biological process of interest (for example, a toxicological or disease endpoint or outcome) but which is too difficult to directly measure itself. Chemical and biochemical biomarkers are generally measured in tissues (including hair and nails) or body fluids. The fidelity with which biomarkers mirror the endpoints of interest varies widely. Well-known examples include the measurement of mercury (or other metals) or drugs in hair or nails (as an indicator of whole-body exposure) and blood pressure and serum cholesterol (as indicators of vulnerability to heart disease). Some biomarkers are prognostic - - indicators of the potential for developing disease, or even disease that has yet to manifest with outward signs. For health conditions with linkages to the environment, prognostic biomarkers and vulnerability biomarkers could be used to guide the design of remedial actions in the immediate environment to prevent or lessen the probable onset or worsening of pathologies. The utility of biomarkers is not just in diagnosis, but also in guiding the selection of subsequent assessment studies for measuring the effectiveness and progress of interventions or other remedial action.

Major limitations in the conventional measurement of any biomarker are the demands on clinical and laboratory resources and the time required for obtaining samples from statistically sufficient numbers of individuals in order to extrapolate conclusions to entire communities or populations; additional hurdles are in gaining human-subject testing approvals. Historically, biomarkers clearly have a number of major limitations in studies at the higher levels of populations or communities for establishing (at a minimum) exposure profiles.

The characteristics of useful biomarkers, partly adapted from Hagger et al. (2006), include those summarized in Table 1. The sewage matrix adds considerable complexities to nearly all of these.

Each of these factors and others would eventually need to be examined before a suitable biomarker (or suite of biomarkers) is selected for measuring community-wide stress using BioSCIM. These are summarized in Table 1. These factors place additional restrictions on the utility of a biomarker, further narrowing an already rather restricted field of possibilities. The numbers of bona-fide biomarkers - - those with accepted clinical utility - - are extremely limited to begin with. Despite the ever-expanding published literature on biomarkers (many resulting

from the various fields of omics), the number of biomarkers that have been validated for use in routine clinical practice may be fewer than 100 (Poste, 2011).

A comprehensive examination of the fidelity with which biomarkers actually measure disease is presented by Ioannidis and Panagiotou (2011); also see Bossuyt (2011). This study revealed major shortcomings in the purported utility of a range of select biomarkers (for roughly two dozen risk factors) for use in diagnosis, assessing vulnerability, establishing prognosis, or guiding treatment. The markers examined were all proteins, genes, or chemicals such as estradiol (which have multiple origins). None of the markers, however, included a biomarker that was amenable to measurement in sewage or that reflected general modes of disease development, such as inflammation (with the possible exception of C-reactive protein: CRP). One controversy surrounding the demonstration of biomarker fidelity with disease is whether large, controlled clinical studies are required (Bacchetti et al., 2011).

7. Effects biomarkers suitable for BioSCIM

In the course of this work, over 900 publications were examined for their possible relevance to different aspects of BioSCIM and to extract supporting data. These articles resided primarily in the fields of clinical research, analytical biochemistry, lipid chemistry, exercise physiology, nutrition, toxicology, and free radical research. One of the primary objectives was to identify the best candidate biomarker with which to develop the initial BioSCIM application. Possible candidates were evaluated on the basis of the criteria in Table 1. The other objective was to use this candidate biomarker to highlight the types of information and data needed to assess biomarkers in general for use with BioSCIM.

7.1. *The Isoprostanes* (IsoPs) - - biomarkers of systemic, system-wide oxidative stress

After evaluating a range of biomarkers as candidates for BioSCIM, the biomarker selected to demonstrate proof of principle belongs to the class of prostaglandin-like isomers - - the

isoprostanes. The isoprostanes (herein referred to generically as *IsoPs*) were first proposed for use as a measure of oxidative stress in 1991 (Morrow and Roberts II, 1991) and have since been accepted in clinical chemistry as the biomarkers that provide the best quantitative measure of total systemic oxidative stress, which serves as a general pathway toward cellular dysfunction (Milne et al., 2008). The biosynthesis of IsoPs was first delineated in 1990, but this new series of prostaglandin-like isomers was not named isoprostanes until 1992 (Morrow et al., 1992; Morrow et al., 1990a; Morrow et al., 1990b).

The IsoPs gained recognition as the most reliable, sensitive, and specific non-invasive measure of in vivo, systemic oxidative stress after a comprehensive series of studies in the early 1990s using rats that were administered carbon tetrachloride. These studies (Kadiiska et al., 2005a; Kadiiska et al., 2005b) composed the first comprehensive comparative assessment of a spectrum of candidate biomarkers of oxidative stress - - the Biomarkers of Oxidative Stress Study (BOSS) of the National Institute of Environmental Health Sciences (Milne and Morrow, 2006). Somewhat analogous studies have since been done with human subjects (Il'yasova et al., 2010).

While the term "oxidative stress" is not rigorously defined, it generally refers to an imbalance in the levels of reactive oxygen, nitrogen, and halogen species relative to the body's defense mechanisms - - a perturbation in the prooxidant-antioxidant balance resulting in disruption of redox signaling and control - - more recently termed "redox disruption" (Breusing and Grune, 2010; Burgos Alves et al., 2010; de Castro Fernandes et al., 2010; Jones, 2006; Jones, 2008); reactive species include both free radical and non-radical oxidants. Indeed, the prevalence of redox disruption versus free-radical oxidation may explain the lack of expected effectiveness of antioxidants reported in many clinical studies.

Oxidative stress originally attracted attention because of the key role it was theorized to play in aging; this theory was an extension of the original "free radical theory" of aging, proposed and advanced in the 1950s by Harmon (see: Kregel and Zhang, 2007). The history of oxidative stress in disease and in maintenance of homeostasis via cellular signal transduction is provided by Hensley and Floyd (2002).

Regardless of its definition, oxidative stress is widely viewed as a hallmark of various acute and chronic diseases, many of which are also part of the normal aging process (Montuschi et al., 2007). Oxidative stress has become recognized as a central factor is the prevalence of chronic inflammatory diseases, including obesity, hypertension, cardiovascular diseases, and type 2 diabetes, among others that define the metabolic syndrome (Jesmin et al., 2010); for all but three of the 15 leading causes of death in the US (Kochanek et al., 2011), inflammatory pathways (including those involving the production of IsoPs) play roles. Note, however, that oxidative stress in itself is not necessarily deleterious, but rather can sometimes be viewed as healthy or necessary - - "eustress"; IsoPs may play roles in governing cellular adaptive response to stress via gene expression and cell signal transduction (Niki, 2008; Noguchi, 2008). A certain controlled, background level of oxidative stress probably plays critical roles in adaptive physiological responses and in critical biological defensive functions (Azzi, 2007). The state of what is known regarding the correlation of oxidative stress and disease has been reviewed by Giustarini et al. (2009).

Although IsoPs are isomers of prostaglandins, they are not directly related, as their mechanisms of in vivo synthesis and their biological functions differ dramatically. The prostaglandins are produced directly in free form by a complex cascade of enzymatically catalyzed reactions (via COX - - cyclooxygenases) beginning with free arachidonic acid (see Fig. 2) - - or any fatty acid with at least three double bonds, notably the essential fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In contrast, the IsoPs are predominantly formed from the non-enzymatic, free-radical peroxidation and cyclization of these same fatty acids only after they have been esterified to cellular membrane phospholipids (at the sn-2 position); note that "peroxidation" generally refers to reactions involving di-oxygenation but some oxidative reactions of lipids involve mono-oxygenation. Oxidation is initiated on the bis-allylic methylene groups (saturated carbon adjacent to two-double-bonded carbons in the *cis* conformation) of the polyunsaturated fatty acid. This non-specific autooxidation results in numerous isomers - - and therefore a complex and often confusing nomenclature.

Structurally, the prostaglandins and IsoPs differ only by conformation (stereochemistry). Each of the three major classes of IsoPs (F-, D- and E-series) can theoretically comprise 64 distinct

isomers; for example, attack of arachidonic acid yields four regioisomers (positional isomers), each of which can yield a further eight isomers, together with diastereomers - - making possible 64 F_2-IsoPs. The two side chains for the prostaglandins are predominantly in the *trans* conformation relative to the cyclopentane ring, whereas they exist in the *cis* orientation for the IsoPs (see Fig. 2); although the more stable *trans* conformation is favored thermodynamically, the more kinetically favored *cis* results for IsoPs because of the bicycloendoperoxide oxidation intermediate.

IsoPs are released in free form from the esterified membrane lipids upon cleavage by phospholipases. In free form, they can then circulate or be excreted (primarily via urine, but also via bile). They can also undergo further metabolism, most notably yielding dinor products (via beta-oxidation); for example, 8-isoPGF$_{2\alpha}$ can be metabolized to 2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$, whose delta-5 double bond can then be reduced to 2,3-dinor-8-iso-PGF$_{2\alpha}$ (Roberts et al., 1996); see Fig. 2. The prefix "nor" (*normal*) refers to removal of one carbon and accompanying hydrogen atom (demethylation or removal of a methylene group); for IsoPs, *dinor* metabolites have two methylenic carbons removed from the carboxy-terminus side chain.

The dinor metabolites can be used as markers themselves, as they serve as surrogates for the parent IsoPs. They are sometimes excreted at levels exceeding those of parent IsoPs by several fold (Burke et al., 2000; Nikolaidis et al., 2011). For the purposes of the BioSCIM concept, the dinor metabolites might be suitable for measuring and summing together with IsoPs to achieve a more comprehensive and larger signal of oxidative stress. Some methods capture all dinor-dihydro metabolites in one chromatographic peak (Davies et al., 2006). Urinary excretion of the dinor metabolites varies depending on the overall beta-oxidation activity. In one study, for example, the ratio of urinary dinor-8-epi-PGF$_{2\alpha}$ to 8-epi-PGF$_{2\alpha}$ was 15 (33.72±5.80 nmol/L versus 2.11±0.41 nmol/L), with 2,3-dinor-5,6-dihydro-8-epi-PGF$_{2\alpha}$ at 16.43±5.62 nmol/L. In contrast, in another study involving 845 women serving as controls in the Shanghai Women's Health Study (SWHS), the levels of the dinor metabolites were roughly 30-50% those of the parent F_2-IsoP (Dorjgochoo et al., 2011); but in a related SWHS study, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP was found to be a more sensitive and specific measure of oxidative stress than the parent 15-F_{2t}-IsoP (Dai et al., 2009; Dai and Zhu, 2009). As the level of oxidative stress increases, the

relative levels of dinor metabolites can decline if beta-oxidation becomes impaired. This variability supports a rationale for measurement of the parent IsoP together with its dinor metabolites (Nourooz-Zadeh et al., 2006).

IsoPs are viewed as providing an integrated measure of systemic oxidative stress because they are produced in every tissue of the body - - as a direct consequence of numerous processes that generate oxidative radicals; they are even excreted via the breath (Janicka et al., 2010). Large quantities are excreted via urine, where the concentration can be 30-40 times higher than in plasma. The different series and isomers of IsoPs can all be formed in different and varying proportions - - and metabolized at different rates. Even though their formation is predominately by free-radical oxidation, this process is influenced by the type and anatomical location of the specific oxidative stress. The spatial distribution of the various isomers in the body is thereby also affected.

Although each of the IsoP classes directly reflects the free-radical oxidation of a single species of polyunsaturated fatty acid (e.g., F_2-IsoPs derive from arachidonic acid), it is widely assumed that polyunsaturated fatty acids (PUFAs) are oxidized indiscriminately, and that formation of a given class of IsoPs from the oxidation of one species of PUFAs serves as a surrogate for other PUFAs. Since a significant portion of IsoPs are incorporated into structural lipids (e.g., membrane lipid rafts and caveolae), a reservoir exists from which free IsoPs can be continually liberated; it is believed that once IsoPs incorporated in cellular membranes are peroxidized that the membranes become more rigid, compromising permeability and altering signal transduction (Dietrich-Muszalska and Olas, 2009). This moderates the free concentrations, serving to prevent fast fluctuations in urinary levels. This partly contributes to the utility of urinary IsoP levels as a measurement index. Other characteristics of IsoPs contributing to their usefulness as biomarkers includes their chemical stability, comparatively low variation in intra- and inter-individual variation in excretion rates, and relative resistance of excretion rates to changes in diet and time of day (Cracowski et al., 2002; Il'yasova et al., 2004).

IsoPs can serve dual roles - - not just as passive biomarkers of exposure but also as active mediators or promoters of effects (Cracowski and Durand, 2006; Crankshaw and Rangachari,

2003; Morrow, 2006). IsoPs may play extremely complex roles as both inhibitors and activators in regulating a wide spectrum of biochemical pathways that feed into numerous physiological outcomes (Niki, 2008; Niki, 2009); they can also serve as incidental ligands, such as for eicosanoid receptors (e.g., the thromboxane receptor). While this aspect is far too complex to summarize here, some of the complexity and unknowns surrounding the biochemical functions of IsoPs is illustrated by their complex roles in the functioning of blood platelets (Ting and Khasawneh, 2010).

IsoPs are believed to play major roles in a broad spectrum of acute and chronic inflammatory diseases, all of which may underlie in particular the general process of aging; see section 8: Factors and variables affecting regulation of IsoP production: disease, lifestyle, and genetics. An overview of the inflammatory response in relation to the eicosanoid cascade is provided by Helmersson (2005). IsoP levels serve as an integrated response from the cumulative exposure to many forms of stress, spanning the range from chemicals to non-chemicals (e.g., psycho-social stress). Indeed, an exposure measure that encompasses psycho-social stress (important especially in assessing environmental justice) persists as another unmet need in cumulative risk assessment (Lewis et al., 2011; NRC, 2011). Moreover, as noted by Lewis et al. (2011), "...it is difficult to imagine how the effectiveness of interventions could be assessed effectively without a metric for estimating targeted, cumulative risk reduction." IsoPs play dual roles, as biomarkers of oxidative stress and as mediators of inflammation. They are both effects-based and stressor-based (see Supplementary materials). They also reflect cumulative exposure. The problems, limitations, and caveats regarding selection of biomarkers of oxidative stress are discussed by Halliwell (2009).

A major postulated strength BioSCIM in general (especially when targeting IsoPs) is that it would serve to integrate the effects from all forms of bona-fide exposure. It would not rely on the conventional approach of compiling recollected (and often subjective or inaccurate) exposures from test subjects via self-reporting and historical records; but the interpretation of BioSCIM results could be aided by such exposure data. A major potential confounder of the approach is whether a few individuals among a population may have extraordinarily high changes in excretion levels of a biomarker sufficient to disproportionately influence the collective data from the entire population.

A major objective in the conceptualization of BioSCIM set forth here is to catalyze discussion, debate, and research regarding its feasibility. IsoPs were selected as candidate biomarkers most likely to yield results because of their established use in clinical research and a sizeable body of published data - - a summary of which is presented in this document. In reality, any number of other biomarkers may also prove useful. Ultimately, a spectrum of biomarkers could form the basis of a comprehensive BioSCIM program. These markers could comprise those that integrate exposure/effects across a broad spectrum of biological processes as well as those that reflect unrelated states of more specific stress or disease. IsoPs are presented here in part as an illustration of the evaluation process needed for implementation. The only limitation to incorporating more biomarkers (especially those of more specificity) is whether they meet the criteria in Table 1. IsoPs reflect a widely integrative measure of cumulative and aggregate exposure/effects relative to the whole body.

7.2. Isoprostanes: background

The chemistry and nomenclature of IsoPs are extremely complex. IsoPs also are but a small part of the vast eicosanoid network (those fatty acids containing 20 carbons - - and closely related fatty acids). There are three different chemical naming conventions for the 64 different isomers that originate just from arachidonic acid: the systems of Taber et al., Rokach et al., and Mueller (Nikolaidis et al., 2011). This has added considerable confusion to the published literature and perhaps has also sometimes led to misrepresentation (Mueller, 2011; Murphy and Fahy, 2010). As one example, 15-F_{2t}-IsoP reflects the Taber naming convention (Taber et al., 1997); see Fig. 2. The first number refers to the location of the acyl side-chain hydroxyl group (i.e., 5, 8, 12, or 15). The subscript number after "F" refers to the number of unsaturated bonds in the side chains; other descriptors denote the stereochemistry of the ring hydroxyl group(s). The 5- and 15-series tend to be the most abundant IsoPs because the precursors to the 8- and 12-series are more prone to further oxidation.

15-F_{2t}-IsoP is one of the key isoprostanes studied, largely because it was the first available commercially. It is the same as 8-isoPG$F_{2\alpha}$ and 8-epi-P$GF_{2\alpha}$ and is also called isoprostaglandin $F_{2\alpha}$ type III or i$PF_{2\alpha}$-III (Rokach et al., 1997; Rokach et al., 2004); see Fig. 2. The different "types" are a function of the PUFA from which they are derived: e.g., arachidonic, eicosapentaenoic, or docosahexaenoic acid. Taber's nomenclature follows conventional chemistry rules, conforms to existing prostaglandin naming conventions, and has been accepted by the IUPAC. Some of the naming conventions do not accommodate commonalities among specific IsoPs with respect to biochemical origin or especially with respect to biological activities (Mueller, 2011). Compared with the prostaglandins, where the unsaturated side chains are all *trans* to the cyclopentane ring, the conformation for the IsoPs is exclusively *cis* (see Fig. 2). Succinct and authoritative discussions of nomenclature are provided by Nikolaidis et al. (2011) and by PiŁacik et al. (2002), among others. Note, however, that throughout the following discussion and Supplementary materials, little attempt was made to standardize the variety of naming conventions used in the articles under discussion.

An extensive listing of over 100 IsoPs (common names, systematic names, synonyms, structures, formulas, molecular mass) can be found at LIPID MAPS (2008; 2011). Other types of IsoPs (and IsoP-like compounds) are produced in vivo (e.g., cyclopentenone-based, neuroprostanes, isofurans), but the focus of the work reported here is on the more stable forms of IsoPs that are extensively excreted via urine - - primarily the F_2-series.

Isoprostanes were originally called "iso-prostaglandins" but their origin within the body is totally distinct - - as cyclooxygenase is generally not involved in their formation. Their production is therefore not affected by the medications that can regulate prostaglandin production (i.e., non-steroidal anti-inflammatory drugs [NSAIDs] or glucocorticoids). The in vivo formation of IsoPs in humans was first reported by Morrow and coworkers (Morrow et al. 1992; 1990). Significantly, while IsoPs act as biomarkers of oxidative stress, they can simultaneously also directly act as pro-inflammatory agents themselves. For example, they are potent broncho- and vaso-constrictors. In this sense, as both products and mediators of disease, they are biomarkers of both exposure and effects (sequelae of oxidative stress - - not always harmful and sometimes essential). They may well cause some of the very pathologies that they are used to measure.

While COX-inhibitors (e.g., NSAIDs) repress the formation of prostaglandins, they have no direct effect on the formation of IsoPs, which are produced by a non-enzymatic COX-independent route. IsoPs are formed as racemic diastereomers. An exception regarding prostaglandin formation is urinary $PGF_{2\alpha}$ (as well as ent-$PGF_{2\alpha}$), trans-side-chain products that seem to be formed primarily via the same non-enzymatic pathway used for IsoP rather than from COX. This prostaglandin and its enantiomer are therefore also measures of free-radical induced oxidative stress - - as opposed to markers of COX activity (Yin et al., 2007).

The discovery of IsoPs was first recounted by Morrow and others (Morrow et al., 1990a; Morrow et al., 1990b). Comprehensive reviews that cover all aspects of IsoPs (nomenclature, biosynthesis, chemistry, metabolism, analysis, roles as markers and mediators in oxidative stress and disease, and cell-signaling) began to appear in the 1990s. Among the many excellent reviews are the following, with perhaps one of the more comprehensive being that of Jahn et al. (Basu, 2008; Basu and Helmersson, 2005; Cracowski and Durand, 2006; Dalle-Donne et al., 2006; Fruhwirth et al., 2007; Jahn et al., 2008; Kom, 2007; Milne and Morrow, 2006; Milne et al., 2005; Milne et al., 2011; Milne et al., 2008; Montuschi et al., 2007; Morrow, 2006; Nälsén, 2006; Nikolaidis et al., 2011; Nourooz-Zadeh, 2008; PiŁacik et al., 2002; Praticò, 1999; Praticò et al., 2004; Roberts II and Fessel, 2004; Roberts II and Milne, 2009; Rokach et al., 2004; Yen, 2010; Yin, 2008). Excellent graphics showing biosynthetic pathways are reprinted in many reviews (e.g., Jahn et al., 2008; Morrow et al., 1990a).

Worth noting is that 15-F_{2t}-IsoP (8-iso$PGF_{2\alpha}$) is the most frequently studied IsoP, not necessarily because of any inherent importance, but because of its early and widespread commercial availability as an analytical reference standard. Indeed, other IsoPs and their dinor metabolites may eventually prove more important as biomarkers. Furthermore, prevalence at local sites or release in specific tissues may not necessarily reflect abundance in urine. Urine provides a measure of lipid peroxidation integrated over a prolonged interval. In this review, emphasis was placed on examining the published literature on urinary excretion rather than on levels in plasma or other tissues. Data on plasma levels may not have relevance with respect to the types and

levels of IsoPs that might occur in sewage. Data on fecal excretion (including bile) would also be relevant but is almost non-existent [see section 10.4: Biliary (and fecal) excretion].

A very brief overview of the larger context of lipidomics in which IsoPs exist is useful in appreciating that they also serve in many respects as surrogate measures for many other oxidized phospholipids (oxylipid) biomarkers (for more discussion, see the Supplementary materials). The roles of IsoPs with respect to oxylipids are covered in the comprehensive review of Bochkov et al. (2010). The diverse roles in disease played by the many products of lipid peroxidation have been comprehensively covered in a number of reviews (e.g., Negre-Salvayre et al., 2010; Niki, 2009; Palmieri and Sblendorio, 2007; Spickett et al., 2010).

Many of the complex aspects of IsoPs are not a focus of this paper, especially since they are amply covered in the reviews cited above. For the purposes of this paper, the most important aspect is the urinary excretion of IsoPs - - as it relates to the proposed monitoring of IsoPs in sewage as a fast and non-invasive means to measure the collective contributions of a biomarker of stress from small-area populations.

7.3. Isoprostanes as biomarkers of both exposure and effects

IsoPs serve as retrospective markers of whole-body oxidant injury (e.g., pathophysiological markers of lipid peroxidation) as well as prognostic markers of potential injury (e.g., as mediators of oxidative stress such as involved with cardiovascular diseases). IsoPs serve as both time-integrative indicators and mediators of oxidative stress. They therefore not only have diagnostic value for disease states but also prognostic value for predicting pathologic conditions. IsoPs not only reflect oxidative stress, they are also involved with causing stress (most directly and fundamentally simply by altering the physical structure, integrity, and fluidity of cell membranes by changing steric properties). At the same time, it is important to keep in mind that IsoPs are also involved in a range of normal processes involved with maintaining health and regulating homeostasis (e.g., activation of p53 and other tumor suppressors required in apoptosis). As such, they can be elevated as a result of non-pathological eustress and seemingly

healthy activities (such as pregnancy and exercise), and most basically, by basal metabolism; they can also display reductions in levels during diseased states - - as a result of inhibition of protective basal-level production.

IsoPs integrate information from a very wide spectrum of biological events across the entire body. They can be viewed and utilized as biomarkers of: exposure (actual internal dose of a xenobiotic), effect (response, disease, altered homeostasis, structure, or function), and susceptibility or vulnerability (reflecting stress or status or change in homeostasis or physiological dysfunction - - e.g., polymorphisms). They can facilitate or assist in diagnosis (in medicine, epidemiology, or forensics), prognosis, and therapeutic intervention (Crimmins and Vasunilashorn, 2011). An advantage of biomarkers of stress such as IsoPs is that a portion of the processes they serve to measure can emanate from disease that has yet to manifest itself as a clinical phenotype and could not otherwise yet be detected by clinical assessment.

A partial listing of pathologies in which IsoPs are elevated are summarized in Basu (2007, Table 1); these include cardiovascular disease, hypertension, diabetes, hepatic cirrhosis, obstructive pulmonary disease, obesity, neurodegenerative diseases, and asthma; inflammation, vasoconstriction, and platelet aggregation are common modes of both acute and chronic action. At least a portion of the biological activity of IsoPs is believed to occur as a result of IsoP acting as incidental ligands for certain inflammatory prostaglandin receptors (Song et al., 2009). IsoPs therefore serve as an integrated measure of a wide spectrum of combined disease states as well as non-disease stress, such as poor nutrition, exposure to smoke (Ahmadzadehfar et al., 2006), or other physicochemical stressors such as drugs that cause idiosyncratic reactions (e.g., Lu, 2006). Their production can be reduced by other stressors such as moderate exercise, or increased, such as by over-training (Margonis et al., 2007). Therefore, not all IsoP production results from disease, and their production can be reduced by certain activities; so data have the potential to become confounded. There has also been insufficient research on IsoP metabolism and excretion as influenced by genetics, gender, age, lifestyle, or other non-disease variables. These factors could all add a layer of complexity and unknowns to the interpretation of IsoP levels in sewage.

By targeting a biomarker of general oxidative stress, the following variables are automatically fully accommodated. In risk assessments, each of these variables must normally be modeled, and in the process must account for numerous unknowns: (i) exposure to ALL stressors (chemical and non-chemical), (ii) actual dose from each exposure event, (iii) additive and interactive effects from all stressors (additivity, synergism, antagonism), (iv) metabolic accommodation (induction or upregulation of detoxication pathways), (v) metabolic activation (induction or upregulation of intoxication pathways), (vi) intra-individual and inter-individual variability (genetic, such as single-nucleotide polymorphisms [SNPs], and epigenetic), (vii) prior exposure history, (viii) windows of vulnerability, and (ix) diurnal fluctuations in metabolism and gene expression - - among others.

8. Factors and variables affecting regulation of IsoP production: disease, lifestyle, and genetics

An understanding of a biomarker's synthesis pathways and mechanism of control is needed to appreciate the limitations in interpreting BioSCIM data. Halliwell and Lee (2010) discuss some of the many factors that regulate IsoP production. Various junctures along the pathways of IsoP formation can be affected by different endogenous biochemical processes or exogenous stressors. For example, if esterified IsoP is being continually created by oxidative assault but the phospholipases have been inhibited, then IsoP levels in plasma (and therefore presumably urine) could decline (with a concomitant accumulation of lipid-bound IsoPs in cell membranes). Likewise, if beta-oxidation processes were to be inhibited (thereby inhibiting the formation of dinor metabolites), free IsoP levels could rise. The major question is whether the rate of IsoP production accurately reflects the rate of lipid peroxidation. In reality, the free IsoP production rate may vary wildly among individuals as a function of a broad spectrum of variables - - not the least of which is what type of stress each is sustaining and whether the stress is acute or chronic.

Studies have examined possible associations of IsoPs with a wide spectrum of diseases - - as both passive and active markers. A number of articles provide summaries (e.g., Schwedhelm et al., 2007). Many studies, especially large epidemiological studies, have targeted multiple

biomarkers and multiple risk factors. This makes it difficult to provide straightforward summary of data on the association of IsoP levels with particular diseases. An example of a larger-scale study comes from the Framingham Heart Study, where urinary 8-epi-$PGF_{2\alpha}$ levels (normalized to creatinine) for 2,828 subjects were found to most strongly correlate with smoking, diabetes, and body mass index (Keaney et al., 2003). Corroborating many other studies, a definitive association with gender was shown, where urinary 8-epi-$PGF_{2\alpha}$ levels for women were higher than in men - - by 16%. The strongest association, as also seen in many other studies, was for smoking, where smokers had 65% higher mean levels than nonsmokers. One factor that has shown variable correlations was age, which in this case appeared to be negatively associated. Another example comes from a 299-subject cohort of the Insulin Resistance Atherosclerosis Study (IRAS), where four urinary F_2-IsoPs were correlated with gender, ethnicity, smoking, physical activity, BMI, waist circumference, weight change, and diabetes (Il'yasova et al., 2011).

Understanding the action of IsoPs as facilitators of disease may be hampered by the possibility that their actions are mediated in concert (or in series) with numerous other IsoPs at a variety of receptors and at requisite absolute and relative concentrations at disparate cellular locations. IsoP excretion levels sometimes seem counterintuitive or paradoxical - - increasing as a result of activities generally associated with healthy lifestyles or activities, and other times decreasing as a result of seemingly unhealthy states. One example shows an association between urinary F_2-IsoP and BMI following a U-shaped curve, with the highest urinary IsoP levels resulting at both the lowest and highest BMI values (Narukawa et al., 2011); with the IRAS cohort, urinary F_2-IsoPs were inversely associated with weight gain (Il'yasova et al., 2011). Also worth noting is that randomized, double-blind, placebo-controlled intervention clinical trials that follow IsoP levels are very rare; most studies are observational. In general, even when statistically significant changes in IsoP levels are seen in clinical studies, they are rarely dramatic.

Some representative studies targeted at correlating specific diseases or stress with IsoP levels are summarized in Table 2 and discussed in more depth in the Supplementary materials. The emphasis has been placed on studies that monitored urine levels rather than plasma levels. Also summarized are studies of those variables that may serve to confound the interpretation of BioSCIM data. Many factors can interact, sometimes serving to thwart generalizations;

contradictory studies are not infrequent. Correlations with given factors can also vary among the various parent IsoPs and their metabolites. IsoP levels not only can correlate with disease activity or severity, but also act as mediators of disease progression.

9. IsoP analysis and complications in comparing published data

Of all the factors involved with developing a BioSCIM approach based on IsoPs, the one that would contribute some of the greatest uncertainty is the methodology used for monitoring and chemical analysis. Several factors contribute to this uncertainty, primarily by adversely affecting variance in both accuracy and precision, as well as in impacting decisions as to exactly what chemical species of IsoP should be targeted for monitoring. As emphasized earlier, minimizing analytical variance will prove to be the key in being able to distinguish stress signals from "normal" or "baseline" levels.

Significantly, of the numerous methods reported in the literature and developed for a wide spectrum of tissues and fluids from humans, animals, and plants, none has ever been applied to sewage, which will undoubtedly pose yet additional challenges for sampling and analysis; some of these challenges have already been discussed (Daughton, 2012). **Important to recognize is that no biomarker of stress, disease, or health has ever been targeted in any chemical characterization study of sewage.**

IsoPs are quantified primarily with the use of either mass spectrometry (coupled with liquid or gas chromatography - - LC/MS or GC/MS) or competitive immunoassay (RIA or ELISA); representative LC/ESI-MS/MS chromatograms for some isoprostanes (among others for various prostanoids and dihydroprostaglandins) can be seen in Masoodi and Nicolaou (2006). While the analytical results from these various methods correlate well in some studies (e.g., Carraro et al., 2010; Devaraj et al., 2001), they do not in others (e.g., Callewaert, 2004; Liang et al., 2003). Since ELISA has clearly been the method of choice in terms of cost and speed (a critical attribute of any method intended for widespread, routine use), it would eventually need to be corroborated

by an orthogonal approach, probably involving MS, especially given the bias in immunoassay that can be introduced by cross-reactivity.

The IsoP most targeted in the literature for analysis in urine is 15-F_{2t}-IsoP. The attributes of the F_2-class of IsoPs that make them ideal as biomarkers are summarized by Montuschi et al. (2004), and include: (i) chemical stability, (ii) generation specifically in vivo by peroxidation and little artefactual formation in urine, (iii) low limits of detection can be achieved in various tissues and fluids, (iv) levels increase substantially during oxidative stress, and (v) levels are little affected by lipid content of diet. They also undergo rather fast elimination from the body (Cracowski et al., 2002), and artefactual formation in urine is minimal because of the absence of arachidonic acid (Kom, 2007). The F_2-IsoPs have been shown to be stable in urine for over a week at room temperature (Praticò et al., 1998) or for up to 3-6 months when stored at -20 to -80°C (Ohashi and Yoshikawa, 2000). While these studies indicate that sample transport and storage might not pose problems, the variables added by the presence of fecal materials leaves open the question of IsoP stability is stored sewage (especially with the potential for microbial degradation). This topic has never been evaluated and marks one of the priorities for establishing IsoPs as a target biomarker suitable for BioSCIM.

Acquisition of accurate and reproducible analytical data is complicated by the fact that 15-F_{2t}-IsoP (as well as probably all other IsoPs) undergoes extensive but variable inter-individual glucuronidation during metabolism. To eliminate this source of variability, samples might first need to be treated with glucuronidase prior to analysis in order to release the free parent 15-F_{2t}-IsoP. Enzymatic cleavage of the glucuronide has been shown to yield up to 80% more free 15-F_{2t}-IsoP in urine (Oxford Biomedical Research 2008a); the role of IsoP conjugates has been addressed in more detail in section 10: Isoprostane excretion data. Another challenge, especially in comparing data across published studies, is the unknown degree to which various IsoP isomers (and isobaric analogs such as the prostaglandins) are resolved during mass spectrometric analysis. Some data are undoubtedly biased high because of the overlap of multiple isomers in a chromatographic peak comprising what might be mistakenly thought to represent a single isomer.

Yet another factor that complicates the intercomparison of published data on urinary levels of IsoPs is the numerous dimensional measurements used to express concentrations. These include both mass and molar dimensions, expressed in terms of either absolute levels (such as mass or moles per unit volume) and relative, dimensionless levels (generally normalized to creatinine); the use of creatinine in clinical chemistry poses a variety of its own problems and would introduce yet more variability for BioSCIM data (Daughton, 2012). Unlike clinical use, where a biomarker's urinary concentration is often normalized to creatinine (to compensate for urine dilution - - a problem caused solely by reliance on spot urine samples instead of 24-h samples), the value relevant to modeling anticipated levels of a biomarker in sewage is the **total** per capita excreted **quantity**, expressed on the basis of absolute mass or moles. Values normalized to creatinine are problematic to accurately translate into absolute rates of daily excretion. This problem can be most readily seen with the gender discrepancies often noted for urinary IsoP levels, where levels normalized to creatinine are usually higher in women. This may actually represent an inherent bias introduced by the fact that women usually possess less lean muscle and therefore excrete less creatinine (Daughton, 2012). Unfortunately, most studies express urinary IsoP excretion normalized to creatinine rather than on a volume-concentration basis; this confounds the interpretation of IsoP excretion data (as well as data for endogenous biomarkers in general) for the purposes of BioSCIM.

A major problem confronting IsoP analysis is the lack of a validated, standardized method, although a method standardized for urine could possibly be modified and adopted for raw sewage. Method standardization is an unmet need with most biomarkers. An example of a major aspect of standardization (inter-laboratory, inter-method comparison) is shown by the work of the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA) for the urinary biomarker of oxidative stress derived from DNA - - 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Evans et al., 2010).

Currently, two major approaches for IsoP analysis involve immunoassay and hyphenated versions of mass spectrometry. A number of commercial immunoassays are available (such as those from Cayman Chemical, Detroit R&D, Enzo Life Sciences, Kronos Science Laboratory, Northwest Life Science Specialties, Oxford Biomedical Research, and OxisResearch), few of

which have been inter-compared or compared versus mass spectrometric methods. Numerous methods using GC/MS or LC/MS have been published, but all have been separately developed for disparate research programs in a variety of fields. Nearly all methods rely on some form of prior separation or cleanup, with a wide spectrum of specificities - - ranging from solid phase extraction or thin-layer chromatography to immunoaffinity chromatography (IAC). IAC for IsoP analysis was first explored in the 1990s (Bachi et al., 1996). IAC has shown potential in sample preparation for providing highly standardized, intercomparable data as a result of its very high specificity for targeted IsoP analytes (Sircar and Subbaiah, 2007; Tsikas, 2010b; Tsikas et al., 2003). Artefactual interferences are problematic for all IsoP methods not using IAC, even when using internal standards (e.g., Mas et al., 2010a). Only more recently has effort been devoted to developing methods (generally based on HPLC) requiring minimal sample preparation (e.g., Saenger et al., 2007).

Comprehensive inter-method or inter-laboratory comparisons are rare. Comparisons of methods based on immunoassay with those based on mass spectrometry are particularly lacking (e.g., Tsikas, 2010a). Poor correlation or high bias has been discussed in a number of studies (Bessard et al., 2001; Huang et al., 2002; Il'yasova et al., 2004; Klawitter et al., 2011; Liang et al., 2003; Saenger et al., 2007; Smith et al., 2011). Some of the possible causes of variability and some caveats regarding sampling, sample preparation, and analysis have been discussed (e.g., Callewaert, 2004; Jones, 2005).

The questionable precision and accuracy of the commercial immunoassay kits (versus MS-based methods) greatly diminishes the possible utility of a large portion of the published literature, especially clinical research that has heavily relied on the convenience of these kits. Inadequate sample purification and cross-reaction among IsoPs are major limitations. Even when good correlations between immunoassay and MS methods are reported, the immunoassays are always found to yield results biased high - - by at least 100% (Carraro et al., 2010; Yan et al., 2007).

A number of comprehensive reviews on IsoP analysis are available (e.g., Berdeaux et al., 2006; Davies, 2009; Lawson and FitzGerald, 2002; Liang et al., 2003; Liu et al., 2009; Milne et al., 2007; Nikolaidis et al., 2011; Yin, 2008). Advancements in the trace analysis of IsoPs in

biological matrices have been infrequent. One analytical approach, developed by Eggink et al. and further developed by Kretschmer et al., makes use of a new derivatization reagent for HPLC (Eggink et al., 2010; Kretschmer et al., 2011). Progress is being made toward automating IsoP analysis, via HPLC/MS (Bai et al., 2011). Others are working on increasing the sample throughput and accuracy of methods (e.g., Dahl and van Breemen, 2010; Langhorst et al., 2010).

Regardless of the dearth of research on method comparability, the various F_2-IsoPs seem to be able to proxy for each other. This is important with respect to another complication associated with analysis - - namely, the separatory resolution of the numerous F_2-isomers possibly present. The actual scope of an F_2-IsoP method is often not clear - - it is sometimes unknown whether a method might be specific for a single isomer or whether it is capturing multiple isobaric species. With that said, it has often been proposed that it might prove more useful to purposefully capture multiple F_2-isomers together (or better, all F_2-IsoPs *in toto*, or perhaps even include F_2-dinor metabolites) in a single analysis rather than target discrete isomers. This approach would greatly improve method sensitivity and lessen variability. This important topic has been emphasized in many papers (e.g., Cracowski et al., 2002; Halliwell and Lee, 2010; Mori et al., 1999a; Nikolaidis et al., 2011; Schwedhelm et al., 2007; Taylor et al., 2008; Tsikas et al., 2003).

Uncertainty in exactly which isomers are captured by a method is often indicated with the use of generic expressions such as "F_2-isomers". A conflated class-wide measure would increase the sensitivity of the method and ensure that the origins of oxidative stress are more widely represented. This is especially true since current knowledge is insufficient to determine which isomer(s) best reflects oxidative stress, which is the most potent mediator of stress, or which is most abundant in urine (Cracowski et al., 2002). Moreover, the relative contributions from individual isomers may change with time, depending on the health status of an individual (individual F_2-IsoP isomers may originate from different diseases and may have different biological effects), and the different isomers may experience different metabolic clearance rates. Regardless, the fact that different methods capture different types and numbers of F_2-IsoP isomers can serve to inflate or deflate the apparent range of urinary IsoP values far beyond what would otherwise be achievable with a standardized method.

An extremely broad array of methodologies and QA/QC have been employed in IsoP analysis. The most important insight to gain from the extensive published literature involving IsoP analysis is that data from different studies (even those using the same basic analytical approach) should probably not be inter-compared with respect to absolute concentrations. The value of these data with respect to BioSCIM currently resides in the perspective they provide on the precision (and the maximum range of values) that might be expected from any given method.

10. IsoP excretion data

Despite the disparities in the way published data on urinary IsoP levels are reported, these data are valuable for determining the potential utility of IsoP in a BioSCIM application. These data address two major unknowns: determining (i) how broad a reference interval might be for urinary IsoP levels from a "normal" population, and (ii) the magnitude of the excursions outside the reference interval for urinary IsoP levels from "non-normal" (e.g., diseased or stressed) individuals. The latter must be significantly greater than the former to be detected.

While the magnitude of the range of data from an individual study is useful, the magnitude of the range for data compiled across studies, which would probably be very large, is not meaningful because of significant differences in analytical methodologies. The broad range in inter-study data distribution is primarily biased by the inclusion of different IsoP isomers in different methods; this is especially evident in the discrepancies between data from immunoassay and mass spectrometric methods.

Much of the IsoP data collected from clinical and epidemiological studies has used immunoassay. These data may be biased high because of cross-reactivity with numerous other isomers and may have larger variance because of intra- and inter-method variability.

A large portion of the IsoP studies conducted with human subjects targeted IsoP levels in blood (generally plasma or serum). IsoPs in blood could be viewed as mirroring acute (real-time) overall stress, while urinary levels serve to integrate stress over time (both acute and chronic).

Blood levels are not necessarily relevant with respect to predicting the levels or variance of IsoP excretion in urine (or feces), as the distribution and metabolism of IsoP is complex. Plasma levels are not necessarily reflected in urinary levels (Halliwell and Lee, 2010). Therefore, the focus here is almost solely on urinary excretion of IsoPs. Another limitation of the published data is that most are obtained from studies that collected spot (convenience) samples, as true 24-h samples are difficult to obtain. Diurnal variability in excretion will mean that spot sample data is more difficult to extrapolate to levels that might be expected in sewage.

As previously emphasized, the actual identity of the IsoP(s) targeted in various studies is often unclear and can range from one to multiple isomers among one or more series of IsoPs. For this reason, the term isoprostane (or IsoP) or other informal abbreviations (e.g., F_2-IsoP) are often used in this document in a generic manner, while fully realizing the lack of rigor. Failure to distinguish IsoP isomers could be a source of considerable variability when comparing data across studies. As one example, in a study that targeted five individual IsoP isomers, the range in urinary levels across the isomers spanned nearly an order of magnitude: from 0.532 µg/day (for 15-epi-iP$F_{2\alpha}$-III) or 0.592 µg/day (for iP$F_{2\alpha}$-III) up to 5.169 µg/day (for iP$F_{2\alpha}$-VI) (Yan et al., 2007).

Another factor treated irregularly across studies is the importance of IsoP conjugates in urine. Excretion of conjugates apparently varies between individuals - - ranging from little up to 80% of the total IsoP present (Kadiiska et al., 2005b; Yan et al., 2010). Although some studies report the use of a pretreatment hydrolysis step using glucuronidase, others make no mention of whether measurement was made for free or total IsoP. Conjugates, however, may not actually have much impact with respect to acquiring data for BioSCIM. Even if substantial portions of IsoP are excreted as glucuronide conjugates, it is possible these will undergo in situ hydrolysis by the glucuronidase activity present in sewage. While most studies show glucuronidase activity in activated sludge, little attention has been paid to raw sewage (on during transit to an STP) or even once feces and urine mix in the toilet. One study, however, shows that estrogen conjugates undergo hydrolysis while in transit to an STP (D'Ascenzo et al., 2003).

The published urinary data from "healthy" subjects (e.g., controls) conclusively show the continual presence of IsoPs in all urine samples. These basal levels may not exclusively represent oxidative damage per se, but rather perhaps serve as lipid messengers involved in myriad endogenous biochemical processes. A certain low basal level of oxidative stress is probably required to maintain homeostasis and health; this is one hypothesis often put forth to explain the "antioxidant paradox" - - the failure of antioxidant supplementation to reduce oxidative stress in healthy individuals (Sheikh-Ali et al., 2011). For example, in the Shanghai Women's Health Study (nearly 75,000 women), supplementation with antioxidants reduced IsoPs only for those with higher BMIs (Dai et al., 2009; Dai and Zhu, 2009).

The production of elevated IsoPs is often a function of current health status and the combined stress level. For example, healthy young adults often show no reduction in IsoP levels when administered fruit/vegetables or antioxidants. This is likely because their IsoP levels are already at the necessary basal maintenance level. Likewise, they often also show no increase in IsoP levels under mild stress (smoking, moderate exercise) because their oxidant defense systems are robust. This means that with a sufficiently low limit of detection, IsoPs should always be detectable in sewage. The critical question is whether the increased IsoP levels in sewage, resulting from oxidative stress, will be sufficiently elevated from basal levels to be able to discern community-wide stress. While any given level of IsoP in sewage most likely results from the combined contributions from many different stressors and causes, a change in trend might more probably result from just a few or perhaps a single cause.

Some studies do not reveal a correlation between known oxidative stress and IsoPs in urine. This is perhaps because the levels in urine are an integrative measure of oxidative stress across the entire body. Localized stress will not always result in increased urinary levels. For example, while exhaled breath condensate shows elevated IsoP levels among certain asthmatic children, IsoP in urine may show no correlation (Carraro et al., 2010).

Despite the considerable published data on urinary levels of IsoPs (for both healthy and diseased subjects), the only values that can be applied directly to predicting the levels of IsoPs that might occur in raw sewage are the 24-h levels (expressed in terms of mass excreted per day). Most data

on the urinary excretion of IsoPs are reported in units that cannot be reliably converted to total daily mass (for example, because of normalization against creatinine or because of reporting on the basis of concentration). **With this said, at least seven studies have reported on the basis of total mass excreted daily**. These urinary excretion data represent basal levels of IsoP production from healthy individuals and span a combined range from roughly 500-5,000 ng/day, depending on the IsoP isomers captured by analysis; most are in the range of 1,000-2,000 ng/day (see in particular: Frost-Pineda et al., 2011; Mori et al., 1999b; Shi et al., 2007; Stein and Leskiw, 2000; Tsikas et al., 1998; Yan et al., 2007; Yan et al., 2010). Since this range comprises extremes from different methods, it could be assumed that use of a standard method would have probably yielded a narrower range.

Sporadically, some data fall outside the commonly reported range. One example is the reported daily urinary excretion for 8-isoprostaglandin $F_{2\alpha}$ at 10 pmol/24 h (3.5 ng/day) (Tsai et al., 2009), which is over 2 orders of magnitude below the low end of the range compiled from the seven studies reported above (i.e., 500-5,000 ng/day).

On the basis of creatinine output, levels of F_2-IsoPs in normal human urine are reported to range from 500 to 4,000 pg/mg of creatinine (Durand et al., 2011), but these data cannot be reliably translated into total daily outputs. They do, however, reflect an inter-method combined range in the distribution of excretion levels similar in magnitude to that for total daily excreted mass (i.e., 500-5,000 ng/day). This shows that the published data have internal consistency.

One study is unique in that it reports excretion data on the commonly used basis (normalized against creatinine) as well as both in terms of urinary concentration (pg/mL) and in terms of mass excreted per day (µg/day). These unique data serve to illustrate an important point. For certain IsoPs, correlations that might not be evident when excretion is normalized against creatinine (e.g., similar levels for smokers versus nonsmokers) become apparent when the data are expressed on another basis, such as in terms of mass excreted per day (Yan et al., 2007).

10.1. Examples of the maximum magnitude of urinary IsoP levels

The conditions under which the maximum urinary IsoP levels have been reported may have value in delineating the upper limits to variation in BioSCIM data resulting from changes in a

community's health status. These reported, enhanced IsoP levels can be roughly one to two orders of magnitude over basal levels.

Perhaps one indication of a potential maximum magnitude of change in urinary IsoP levels that might result from oxidative stress was shown in the original BOSS report, where urinary 8-iso-$PGF_{2\alpha}$ and 8,12-iso-$iPF_{2\alpha}$-VI increased 10 and 17 fold, respectively, 7 h after treatment with the highest carbon tetrachloride dose (Kadiiska et al., 2005a). Such a dramatic response, albeit in rats, might make detection possible even with a high level of baseline (basal) variability.

Probably the highest reported rates or changes in rates for IsoP excretion in humans have been documented for genetic disorders. These excretion rates might serve to describe the absolute upper limit on IsoP excretion across the numerous other disease states known to influence urinary IsoP. One example studied children with Zellweger syndrome - - a rare genetic disorder preventing the proper assembly of peroxisomes and leading to the accumulation of very long chain fatty acids in the blood. Five Zellweger children were shown to excrete 8-Iso-$PGF_{2\alpha}$ at rates over a 100-fold higher than healthy controls: 63.3±16.6 ng/mg creatinine versus 0.51±0.16 ng/mg creatinine (Tsikas et al., 1998).

Although considerable data have been obtained on the correlation of IsoP production in humans with various disease states, few data are available on the production of IsoP as a result of induced stress such as with the rodent models used in the BOSS assessment. One of the few occasions for acquiring such data is during chemotherapy infusion. One study followed the urinary production of four isomers of F_2-IsoPs [iPF(2α)-III, 2,3-dinor-iPF(2α)-III, iPF(2α)-VI, and 8,12-iso-iPF(2α)-VI] during doxorubicin infusion. After 1 hour, the increase in levels ranged from 41% [iPF(2α)-VI] to 62% [iPF(2α)-III]; all levels returned to baseline after 24 hours (Il'yasova et al., 2010). This type of study is valuable in that it gives insight regarding the magnitude of increase in urinary IsoPs that can occur after a significant oxidative insult. It also provides insight on the speed and sensitivity of IsoP production and rate of return to baseline. The fact that IsoP returned to baseline levels before doxorubicin had been cleared from the body perhaps meant that antioxidant defense mechanisms had been quickly up-regulated. In a follow-up doxorubicin study involving 18 women, the levels of the four isomers in 10 subjects nearly doubled. It was

also shown that for others who began the study with already elevated IsoP levels, doxorubicin had little effect (Il'yasova et al., 2011).

Of all common stressors, one responsible for some of the largest increases in urinary IsoP levels is chronic alcohol consumption associated with liver disease. For healthy adults, urinary excretion of $iPF_{2\alpha}$-III initially increased about 4-fold from baseline [from 116 pg/mg creatinine to 491 (0-6 h) and 349 (6-12h)] and then declined to 202 (12-24h) after dosing with 0.9 gm/kg alcohol. The levels were also a linear function of dose, increasing from baseline (50 pg/mg creatinine) to 102 at a dose of 0.2 g/kg alcohol, and to 402 for 0.9 g/kg alcohol (Meagher et al., 1999). Urinary levels were higher in patients with acute and chronic alcohol-induced liver disease (ALD) (657 pg/mg creatinine) and higher yet in patients with combined hepatitis C cirrhosis and ALD (922 pg/mg creatinine versus a baseline of 127 for healthy controls). The highest levels were from those with acute alcoholic hepatitis (2,205 pg/mg creatinine). This represented roughly a 20-fold increase over controls - - one of the largest increases published to date. Excretion of $iPF_{2\alpha}$-III was found to be highly correlated with $iPF_{2\alpha}$-VI, which occurred at much higher levels. The metabolite of $iPF_{2\alpha}$-III (2,3-dinor-5,6-dihydro-$iPF_{2\alpha}$-III) was also markedly elevated.

10.2. Example urinary IsoP ranges for apparently healthy populations

Few studies have been devoted to establishing urinary IsoP levels for "healthy subjects" or perhaps more appropriately termed "apparently healthy" or "otherwise healthy" subjects. The primary source of data for apparently healthy individuals must usually be derived from their use as controls in clinical studies. Once again, the primary relevance of these studies for a BioSCIM application is not the absolute levels reported, but rather the variance that is revealed. But even with studies of apparently healthy individuals, considerable variance is undoubtedly introduced simply by the prevalence of common factors known to influence IsoP levels - - the very factors that would ordinarily be reflected by BioSCIM - - especially smoking, alcohol consumption, exercise, BMI, and age - - as well as any of numerous potential sources of hidden stress. No published studies enlisting otherwise healthy individuals were found that controlled for all of these major variance factors, either during recruitment or by statistical means.

Some example data from various studies are briefly summarized here to provide perspective on the magnitude of variance encountered. In 323 apparently healthy Japanese, mean urinary 8-IsoP was 0.74±0.03 ng/mg creatinine. Levels were significantly higher in males than females and increased with BMI and frequency of alcohol consumption (Sakano et al., 2009). A study specifically designed to determine a "reference interval" for urinary 8-iso-$PGF_{2\alpha}$ enlisted 34 healthy subjects. The range was 57 to 390 ng/g creatinine with a mean of 221 ng/g creatinine (Saenger et al., 2007). In a study of 72 healthy young women and men (36 each), urinary F_2-IsoPs were found to be invariant (2.26±0.9 µg/g creatinine) (Burgos Alves et al., 2010). In a study of 1,647 women (Study of Women's Health Across the Nation - SWAN) spanning the menopause transition, mean urinary $F_{2\alpha}$-IsoP levels in non-smokers did not differ for pre- and post-menopause: 343±12.4 pg/mL versus 379±19.5 pg/mL (Sowers et al., 2008). In the SWAN study, 1,610 participants (multi-race/ethnic sample of midlife women) had an overall median concentration of urinary $F_{2\alpha}$-IsoPs of 433 ng/L, with an overall total range spanning over an order of magnitude: 167-2,074 ng/L (Tomey et al., 2007). Urinary 15-F_{2t}-IsoP from 16 apparently healthy individuals ranged from 55 to 348 ng/g creatinine (Haschke et al., 2007).

One study compared 30 subjects with untreated metabolic syndrome against 30 age- and gender-matched controls. Mean urinary F_2-IsoPs (pmol/mmol creatinine) were 808 (695-943) versus 664 (590-749), respectively (Tsai et al., 2009). In a study using 11 clinical urine samples (unspecified origin), the distribution range for 8-isoPGF_2 was wider and higher than for eight samples from healthy controls: mean and median of 0.118 and 0.092 ng/mL (range 0.029 to 0.240 ng/mL) for clinical samples versus 0.048 and 0.039 ng/mL (range 0.017 to 0.084 ng/mL) for controls (Bai et al., 2011). Just for perspective, of the numerous studies reporting on blood levels, "normal" concentrations of F_2-IsoPs in human plasma were reported as 35±6 pg/mL (0.035 ng/L), versus 1.6±0.6 ng/mL in human urine (Milne et al., 2007).

Some studies, however, yield rather large ranges. In a study of 246 women, the range for baseline urinary levels of 8-iso-PGF_2 was 192-4,873 pg/mg creatinine (Thompson et al., 2005).

A multi-country study used 588 subjects from Sweden (n=220), Italy (n=203), and Poland (n=165). Modestly higher levels were found for smokers. Mean levels (and ranges) for urinary

F_2-IsoPs (pmol/mmol creatinine) among all subjects were 200 (64-1235) [n=588]. For individual groups, the levels were: 182 (64-1235) [n=217] non-smoking males; 213 (70-677) [n=89] smoking males; 204 (68-449) [n=195] non-smoking females; and
245 (116-752) [n=81] smoking females (Basu et al., 2009).

10.3. Intra- and inter-individual variation in urinary IsoP levels

Studies on temporal variation of urinary IsoP levels have examined intra- and inter-personal excretion over periods ranging from days to seasons. Among the first studies to examine intra-day variation in urinary IsoP excretion in healthy subjects were those of Helmerson and Basu. Urinary 8-iso-$PGF_{2\alpha}$ levels (determined by immunoassay) were indistinguishable during a 24-h sampling for each of 10 healthy subjects, with a mean level of 0.44±0.23 nmol/mmol creatinine (Helmersson and Basu, 1999). For 13 healthy males and females, mean urinary 8-iso-$PGF_{2\alpha}$ on 10 consecutive days was 0.27±0.11 nmol/mmol creatinine (Helmersson and Basu, 2001).

Using a method developed specifically for an 8-iso-prostaglandin $F_{2\alpha}$ dinor metabolite (2,3-dinor-$iPF_{2\alpha}$-III), a study of 21 subjects measured intra- and inter-individual variation in urinary levels (Zhang et al., 2010). The overall mean was 4.3±0.3 μg/g creatinine. As with many studies, the mean levels were significantly higher for females than males (5.0±0.6 versus 3.6±0.3 μg/g creatinine). Respective intra- and inter-individual contributions to total variation were 40% and 60% (Zhang et al., 2010).

One of the few and most significant studies on time-course variability of urinary IsoPs was a year-long study of 48 randomly selected middle-aged and elderly Chinese men (Wu et al., 2010). Spot urinary samples were collected over 4 seasons. Among the markers targeted were F_2-IsoPs and 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (15-F_{2t}-IsoP-M). The mean levels (expressed as the mean interquartile range) for each of the four seasons were 2.21, 1.88, 1.89, and 1.80 ng/mg creatinine for F_2-IsoPs; the grand mean was 1.90. For 15-F_{2t}-IsoP-M, the mean interquartile ranges were 0.55, 0.53, 0.51, and 0.52 ng/mg creatinine (grand mean of 0.53). These data reflected surprisingly low variation and high stability among the seasonal levels, especially given the use of spot instead of 24-h samples.

10.4. Biliary (and fecal) excretion

In examining published data for the levels of a biomarker projected to be excreted from healthy and diseased individuals, urine is usually the primary route considered - - primarily because of the ease of sampling urine in clinical studies. But the parallel route of fecal excretion (via bile and possibly by intestinal excretion [diffusion into the lumen]) also needs to be examined for BioSCIM. The possibility of two routes of excretion (urinary and fecal) could also dictate the need to design different approaches for sewage analysis depending on whether a portion of the targeted biomarker is not dissolved or suspended in the aqueous phase or raw sewage, but rather remains sorbed to the solids. With some biomarkers - - coprostanol being one example (Daughton, 2012) - - excretion via the feces can be substantial. For IsoPs, however, few data are available on fecal excretion. It is also not known whether feces could also harbor IsoPs that are created within (but not absorbed from) the gut (as with coprostanol).

In the first study of biliary excretion in humans, esterified F_2-IsoPs were found to be excreted in the bile of healthy individuals at levels of 188±27 pg/mL. In subjects with bile duct stones and various diseases of the pancreas, the levels were nearly 3-fold higher - - 523±129 pg/mL and 545±112 pg/mL, respectively (Leo et al., 1997). Assuming an average daily bile production rate of 600 mL, the total daily elimination of F_2-IsoP via the feces for healthy individuals could amount to roughly 113 ng/day. This amount may prove to be significant compared with urinary excretion - - contributing from 23% to 2% of the combined urinary range reported earlier (500-5,000 ng/day); see section 10: IsoP excretion data. The only prior study on biliary excretion used rats, where biliary excretion was found to greatly increase upon dosing with carbon tetrachloride, and most IsoP was excreted in esterified form rather than free (Awad and Morrow, 1995).

11. *Differential Stress Index*: Increasing the sensitivity of BioSCIM by also measuring biomarkers of health to derive a normalized index of stress or homeostasis

One of the major challenges facing the use of BioSCIM for assessing community health is the need to accurately know the size of the contributing population. The population size allows calculation of per capita contributions to biomarker levels measured in sewage. Sufficiently

accurate estimation of population size, however, is fraught with difficulties. One possible approach (ASAP-SCIM: see Daughton, 2012), which for the first time makes use of biomarkers in sewage, has been published as the prelude to this article, but the approach still poses a number of challenges for successful verification and implementation; this published paper also discusses the many limitations to the existing approaches for estimating population size. Any additional sources of error beyond that involved in biomarker analysis and calculation of sewage flow could obscure the ability to detect otherwise significant variations in biomarker levels.

Since the need to calculate community-wide contributions of biomarkers on a per capita basis is problematic, an alternative approach would greatly help. One possible approach (referred to here as the *Differential Stress Index*) would nullify the need to know the specific population size responsible for a measured biomarker level. This approach would create a dimensionless ratio by normalizing the levels of the targeted biomarker of stress (such as IsoPs) against a second biomarker with orthogonal characteristics. The second biomarker (the denominator) would be selected so that its excreted levels would move in opposition to the level of the biomarker of stress (the numerator). To maximize the sensitivity of the Differential Stress Index, the second marker would ideally serve as a positive measure of health.

Dimensionless ratios are routinely used in testing for abused drugs in sports, an example being testosterone/epitestosterone (Van Renterghem et al., 2010). Another example is a proposed "index of endogenous anti-inflammatory potential," which uses the ratio of two oxylipids having opposite effects on inflammation (Gangemi et al., 2005).

Excretion of this second "normalizing" biomarker would have to meet one of two criteria. Its excreted levels would need to be either: (1) a constant function of per capita excretion (and therefore serving as a surrogate measure of per capita population), or (2) a variable function of a physiological state whose level is an inverse, orthogonal function of the targeted biomarker.

Two examples of the first criterion are creatinine and the fecal sterol coprostanol. Creatinine, although long used in clinical chemistry for avoiding the problems created by the extent of dilution of urine (which is problematic for interpreting levels in spot samples), has a number of

problems when applied to sewage (Daughton, 2012). A more likely candidate is the use of coprostanol as a proxy for population size, as proposed by Daughton (2012).

An example of the second criterion is easy to conceptualize but identifying a real-world candidate from the universe of known biomarkers proves difficult. The ideal candidate for an inverse, orthogonal biomarker would be one that measures "good health" or "positive health" - - or an array of biomarkers that correlate with the lowest risk of morbidity and mortality.

Biomarkers are often classified according to three purposes: measuring dose (exposure), effects, or susceptibility to effect or risk. Surprisingly absent are biomarkers that directly measure or confirm health or maintenance of homeostasis. The *absence* of disease is invariably used as an indirect surrogate for health; but such a signal is necessarily one of ever-declining magnitude. With very few exceptions, nearly all known biomarkers measure some attribute of disease or stress. The few possibilities of positive markers of health (which increase in magnitude) tend to not meet one or more of the three major criteria for use with BioSCIM (Table 1). In particular, they must be: (1) sufficiently stable to be extensively excreted - - preferably into urine (or possibly feces) - - and persist in sewage, (2) excreted in the absence of stress at levels with low intra- and inter-individual daily basal variation (including not being influenced by normal dietary changes), (3) immune to confounding - - introduced into sewage predominantly as a result of endogenous human metabolism (with minimal exogenous contribution, such as by raw or cooked foods), and (4) have a chemical structure amenable to detection in the complex matrix of sewage.

A biomarker of health would provide an independent variable against which to normalize the levels of the biomarker targeted for measuring disease or stress. The per capita contributions of the two biomarkers (from opposite ends of the health-disease continuum) in sewage would vary in opposing directions. Normalizing a measure of negative health against a measure of positive health (creating a dimensionless index) would not just eliminate the need to know the population size, it would also serve to greatly amplify the signal that would otherwise be provided solely by measuring a biomarker of stress (as a result of the numerator and denominator being inversely related).

11.1. Biomarkers of health - - anti-inflammatory eicosanoids

A clear example of one of the few documented biomarkers of positive health would be one of the many eicosanoids that serve as counter-regulators to the inflammatory oxylipids. The chemistry and biochemical pathways involving eicosanoids are extraordinarily complex and intricately inter-connected. They are also incompletely understood, with new knowledge continually emerging. The counter-regulators of inflammation are involved not just with down-regulating inflammation, but also with "resolving" (or repairing) inflammatory damage (catabasis). This group of anti-inflammatory, pro-resolving eicosanoids comprises the specialized classes of proactive, lipid mediators known as lipoxins, resolvins, protectins, and maresins, many of which are biosynthesized from the omega-3 essential fatty acids eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Even though these anti-inflammatory pro-resolution eicosanoids are produced at highest levels during the recovery phase (resolution) of chronic disease, their presence reflects a healthy immune system; their levels are notably suppressed in those who are unable to resolve chronic inflammation. Overviews are available (Das, 2010; Levy, 2010; Maderna and Godson, 2009; Serhan and Petasis, 2011).

These pro-resolving, hydroxylated, polyunsaturated fatty acids are produced in localized areas of tissues at extremely low concentrations (pM-nM), reflecting their hormonal-like potency. These oxylipids would make ideal biomarkers of health. But unfortunately, little is known about their excretion in urine, and their low levels might challenge analysis. Limited data, however, indicate that certain drugs (e.g., COX-2 and LOX inhibitors) might inhibit the resolution process, whereas others might allow it (e.g., aspirin, statins, and glucocorticoids) (Serhan and Petasis, 2011).

One of the only studies published to date on the urinary excretion of pro-resolving mediators involves a lipoxin, LXA4. Its urinary levels were roughly 2 orders of magnitude lower than those commonly reported for IsoPs - - roughly 0.02 ng/mg creatinine (Gangemi et al., 2005). The only published method for lipoxins in urine is an immunoassay, and it is unknown whether its sensitivity and specificity would suffice for sewage.

11.2. Other potential markers of positive health for use as denominators in the Differential Stress Index

In addition to the pro-resolver oxylipids, several other potential biomarkers of "positive health" exist but for a variety of reasons would not work as the denominator in a differential stress index. The production of some biomarkers reflects dual actions - - serving as indicators of both positive health attributes and negative aspects of disease. Two sometimes paradoxical outcomes can come about from the same mode of action - - sometimes as a function of the concentration or other times as a function of site or timing of production. One example is the production of an array of halogenated lipids from the oxidative stress created by endogenous hypohalous acids. This can result, for example, from collateral damage from immune response in fighting an infection (e.g., Spickett, 2007).

11.2.1. F_3-Isoprostanes

While the major focus of IsoP research has been on the F_2-series, many other IsoPs can be formed from various polyunsaturated fatty acids other than arachidonate. In 1997, the IsoP F_3-series was reported to form from the non-enzymatic free-radical peroxidation of eicosapentaenoic acid (EPA), an essential omega-3 fatty acid whose primarily dietary source is fish oil (Nourooz-Zadeh et al., 1997); additional amounts of F_3-IsoP could also be contributed by beta-oxidation of F_4-IsoP generated by analogous peroxidation of the related omega-3 docosahexaenoic acid (DHA). The in vivo formation of F_3-IsoP was first shown by Gao et al. (2006).

Of the six possible series of F_3-IsoPs (5-, 8-, 11-, 12-, 15-, and 18-series), the most prevalent in urine seem to be 5-epi-8,12-iso-iPF_3-VI and 8,12-iso-iPF_3-VI, whose ratio is relatively fixed (Song et al., 2009).

F_3-IsoPs tend to be produced more efficiently than F_2-IsoPs - - probably being more easily formed thermodynamically since EPA and DHA (having 5 double bonds) are more unsaturated than arachidonic acid (AA) and therefore are more readily oxidized. When EPA and DHA are present, they might therefore serve to competitively reduce the oxidation of AA, resulting in

lower production of inflammatory F_2-IsoPs. Indeed, clinical data show that EPA and DHA can reduce F_2-IsoP levels (Mas et al., 2010b).

Evidence also indicates that the F_3-IsoPs possess their own anti-inflammatory actions (Roberts II and Milne, 2009). Although the generation of F_3-IsoP from EPA is much more efficient than F_2-IsoP from AA, urinary levels of F_2 are substantially higher than F_3, probably because of the much higher levels of AA than EPA (or DHA), whose dietary source is limited primarily to fish oil. Moreover, the intra-individual excretion rates of F_3-IsoPs seem to be much more variable than for F_2-IsoPs. Nonetheless, F_3-levels, if detectable in sewage, might have potential as orthogonal biomarkers against which to normalize F_2 levels.

Other factors can disqualify a biomarker from use with BioSCIM. Some of these are illustrated by the adrenal androgen dehydroepiandrosterone (DHEA), which is one of the few biomarkers tracked in existing clinical tests that purportedly reflects positive health (see the Supplementary materials section "Aspects of biomarkers not suitable for BioSCIM").

12. Consideration of other biomarkers for SCIM

Other biomarkers were considered for use in an initial (proof of principle) BioSCIM application but were determined to have one or more negative attributes compared with IsoPs; many of these also have extensive histories of use in clinical research, such as prostate specific antigen (PSA) and various hormones. Among these are not just other markers of lipid oxidation [nitrated fatty acids; plasmalogens (Leßig and Fuchs, 2009), halohydrins (Spalteholz et al., 2004), and other chlorinated lipids (Spickett, 2007)], but also products of protein oxidation [*o,o'*-dityrosine, nitro-tyrosine, and halo-tyrosines (Mohiuddin et al., 2006)], DNA (oxidized guanine derivatives such as 8-oxoguanine) (Andreoli et al., 2011; Winnik and Kitchin, 2008), and uric acid (metabolized to allantoin) (Il'yasova et al., 2010); for an overview, see Jain (2010). Many of the halogenated biomarkers are formed from the action of myeloperoxidase (MPO), which uniquely generates hypohalous acids from halides at physiological concentrations (Klebanoff, 2005).

While it might seem that halogenated products could serve to facilitate detection in sewage, the ubiquitous occurrence of myriad organohalogen natural products, particularly in marine life, might confound the use of a halogenated biomarker for SCIM. The occurrence of organohalogens in food sources might serve as a significant exogenous source (Gribble, 2010).

13. Biomarker profiles and community-wide allostasis

The utility of BioSCIM could be enhanced with the incorporation of a select array of biomarkers - - preferably reflecting a wide spectrum of biological regulatory processes and providing complementary, orthogonal measures of disease (or health). It is now widely recognized that aggregate exposure to multiple stressors sharing the same mechanism or mode of action - - but at individual levels below any known effects levels - - can yield adverse effects as a result of their combined contributions. But much less is known with regard to stressors acting by unrelated pathways and yielding unrelated effects (mixed-outcome effects). The question is whether individual effects, which would otherwise not prove adverse in isolation from others, when combined with the unrelated effects elicited by orthogonal-acting stressors, might serve to exceed the allostatic load.

An array of carefully selected biomarkers would better reflect the effects from combined cumulative exposures. Such a composite picture - - or profile - - would more closely align the BioSCIM concept with the *biological passport* approach used in sports random drug testing (referenced earlier). Perhaps the first and most comprehensive attempt at designing a composite measure of how the body responds to cumulative exposure to all stressors is the concept of allostasis and its quantitative measure referred to as allostatic load (McEwen and Stellar, 1993).

In practice, allostasis is measured by the combined levels of roughly a dozen or more biomarkers whose increasing (or decreasing) levels are associated with an increased risk of mortality. Conceptually, allostasis reflects the body's overall status in maintaining homeostasis and indicates when perturbations exceed that capacity. The allostatic load accounts for the two forms of stress: quotidian (ordinary, recurring, persistent, chronic, episodic, daily stress, which tends to be perceived as minor), and infrequent, intense, acute forms of stress. Significantly, allostasis

would also account for health disparities caused by stress resulting from origins usually not reflected by traditional chemical-exposure monitoring - - stressors such as socioeconomic status, ethnicity, race, and psychological stress (Djuric et al., 2008).

A recent review on allostasis is available from Juster et al. (2010), and a study examining the weaknesses of the allostatic load concept is presented by Dowd et al. (2009). A variety of analogous approaches intended for practical clinical application have been proposed (Ochi and Cutler, 2003; Southern, 2010; Veglia et al., 2010).

14. Potential role for BioSCIM in revealing health disparities (via IsoPs)

Health disparities reflect the disproportionate morbidity, mortality, stress, and degraded productivity and sense of well being experienced as a result of differential exposure by racial and ethnic minorities and by the disadvantaged/under-served, such as rural and poor populations. These factors coalescence into greater need for healthcare but which is often not available. Health disparities often result from combined actions of genetic and epigenetic factors, social setting, psychological stress (and impact on well-being), environmental stressors (including diet), lifestyle choices, and insufficient access to medical care (Djuric et al., 2008). Traditionally, an overwrought focus has been devoted to chemical pollutants at the exclusion of numerous other stressors (Morello-Frosch et al., 2011) - - many of which have outcomes measurable by oxidative stress.

The need to assess cumulative impacts of aggregate stressors at the community level is certainly an emerging interest and need. To date, the approach used in assessing cumulative impacts focuses on assessing proximity to known hazards, social vulnerability indicators, land use, demographics, and other signs or measures of possible disproportionate exposures. The variety of methodologies employed have been summarized (Alexeeff et al., 2010; Jakubowski and Frumkin, 2010; Medina-Vera et al., 2010; Zartarian and Schultz, 2010). Some of the many projects underway have been summarized (SEHN/CHE, 2011). BioSCIM could help as a new

tool in assessing cumulative exposure and risk in communities, such as with EPA's Community-Focused Exposure and Risk Screening Tool (C-FERST) (Zartarian et al., 2011).

These methodologies are not quantitative, but rather designed to facilitate relative rankings of communities (e.g., from disproportionate exposure to one or more documented stressors). While conventional measures of stress or disease are used (e.g., known incidence of disease such as cancer), none of the approaches relies on biomarkers of exposure or effect.

An emerging appreciation for health disparities and environmental justice led to the creation of what is now the National Institute on Minority Health and Health Disparities (NIMHD). Needed are quick and inexpensive measures that account for the multi-factorial nature of health disparities - - including those of psychosocial origin (e.g., emotional stress, such as brought about by socioeconomic status). A biomarker or suite of biomarkers that reflects cumulative stress from all sources would be extremely useful. It appears that no research has ever been published on determining cumulative exposures to any class of stressor via biomarkers; a major impediment has been the lack of an approach for collecting samples on a community-wide scale (Lewis et al., 2011). Sexton and Linder discuss the complexities of assessing cumulative risk and the additional challenges posed by non-chemical stressors - - in particular psychosocial stress (Sexton and Linder, 2011).

With the appropriate biomarker(s), BioSCIM holds potential for helping to reveal hidden or emerging disparities, and to assess the effects of attempted interventions. It could help accelerate and improve our understanding of community-wide health disparities. Conventional monitoring of biomarkers in individuals requires considerable resources, and major challenges and limitations are posed by field work (especially in rural areas), all adding to delay in dissemination of data. Conventional health disparities research can suffer from: bias introduced by selection of a limited study population, by accessibility of clinics or in-home clinicians, or by sample processing, storage, and shipping (Djuric et al., 2008).

In an overview of biomarkers (Djuric et al., 2008), a range of biomarkers were examined for assessing health disparities. In the data examined for the work reported here, only IsoPs were

deemed suitable for use in BioSCIM because of shortcomings with one or more of the criteria in Table 1. For example, although baseline plasma levels of 15-F_{2t}-IsoP were reported to not differ between African Americans and white Americans, levels of 15-F_{2t}-IsoP increased more in African Americans in response to acute hyperlipidemia, which is a simulation for postprandial oxidative stress and which may be a factor in ethnic differences in cardiovascular and renal risk (Lopes et al., 2003). In a study of cognitive aging, Caucasians were reported to have lower levels of urinary IsoPs than Hispanics (Insel et al., 2011).

15. Limitations of BioSCIM

A wide array of potential problems could limit the application of BioSCIM, regardless of the targeted biomarker. Foremost among these are problems with the sampling of sewage streams representative of the entire population served by the STP. This aspect has been discussed (Daughton, 2012). The sewage influent stream to an STP at any point in time serves as a sampling of combined excretion from a random sub-population of the total population served by the STP. The relative size of this random sub-population is also unknown. This necessitates the use of continuous in-stream sampling designed to collect the total flux of the biomarker over a sufficiently long time (probably at least a day). This poses a number of challenges with respect to the technologies used in sampling. Moreover, however, despite the sophistication of whatever sampling approach might be used, additional hurdles are faced with respect to the representativeness of the data. These result from the fact that diurnal variations in the excretion of urine and fecal matter, coupled with possible diurnal variations in biomarker excretion, cannot be fully represented in any continuous sampling process simply because a certain portion of the population served by the STP will be absent from the service area at any point in time.

Several additional limitations include factors having the potential for confounding BioSCIM data based on IsoP measurement. These variables range from those with the potential to alter IsoP formation and excretion (in the absence of exposure to a stressor) to those that create analytical artefact. Major variables include age, medications and food supplements, dieting (e.g., fasting), nutrition, sewage contributions from healthcare facilities (such as long-term care facilities and

hospitals, where excreted levels could be expected to be unusually elevated), and exogenous (ex vivo) sources of IsoPs that would inflate the levels resulting from endogenous stress. Corrections could be implemented for some of these; an example would be the age structure of the local population, where perhaps demographics could be used to account for age. Several examples of confounding factors are provided in Table 3; these are discussed in more detail in the Supplementary materials (see section on "Potential Confounders").

16. The future

Even though a BioSCIM application remains to be reduced to practice (whether based on IsoPs or alternative biomarkers of stress or health), foreseeable advancements in various technologies could clearly add further power to the approach. These could range from the mundane and obvious to the esoteric. Most simply, better biomarkers of oxidative stress might well exist (e.g., those with greater excretion rates or more easily analyzed) but perhaps have simply not yet been recognized. The development of a certified reference material (e.g., IsoPs in a matrix simulating urine or sewage) could help eliminate a large portion of the uncertainty deriving from analytical measurements between labs (especially when using differing methodologies and instrumentation).

More advanced developments include development of in-line, automated sewage monitoring capability. With the development of in-stream biomarker sensors that could function in raw, untreated sewage, data could be streamed from sewage distribution lines to web-based community dashboards displaying continuous real-time information showing absolute status or trends. The data generated by BioSCIM could be validated by using the voluminous health data being made available via the Community Health Data Initiative (CHDI), launched in 2010 by the US Department of Health & Human Services (HHS, 2010). These capabilities could eventually also provide the type of instant feedback that has proved so effective in motivating individuals to begin changing their behaviors in ways conducive to achieving continual improvements in health or well-being. For example, the use of feedback and peer-to-peer comparisons (such as via social networking sites) has been shown effective in reducing household energy usage (Foster et al., 2010). BioSCIM data revealing a clear time trend for a given STP could be used as positive

reenforcement for communities possessing healthy lifestyles, or as a means to flag those communities where certain factors may be degrading health; an ability to predict declining community health allows for development of interventions.

The availability of inexpensive sensors that could target IsoPs and other biomarkers would clearly advance the implementation of BioSCIM. Recent advances in sensor technology are bringing this closer to reality. One example is the adoption and reengineering of the ubiquitous personal glucose monitor (used by diabetics) for the detection and quantitation of a broad array of new analytes via the use of functional-DNA conjugated to invertase (which hydrolyzes sucrose to yield glucose) (Xiang and Lu, 2011). Inexpensive monitors could empower local communities to participate in the implementation of BioSCIM.

A more esoteric future possibility would be the development of what might be called "health checks" for entire communities. One approach would be the use of an exogenous chemical probe specially designed to reflect the level of active stress when taken orally - - for example, by way of producing unique metabolites when subject to peroxidation. If a sufficient portion of a community's population participated, then sewage could simply be analyzed for a metabolite unique to a chemical probe and which is indicative of oxidative stress. Indeed, such chemical probes have been proposed for use in clinical medicine (Khatib et al., 2007; Szuchman et al., 2008; Vaya, 2008; Vaya and Tamir, 2008).

TABLES

FIGURES

Table 1. Ideal attributes for biomarkers to target with BioSCIM [a]

biomarker attribute	example	potential limitation
sensitive to changes in amplitude or duration of stress, disease, or health	excreted levels must be a function of the amplitude of adverse or beneficial health effect	some biomarkers can reflect both adverse and beneficial health effects
should be known what physiological system or biochemical pathway the biomarker acts upon or results from	range can span from very specific (a particular organ or process) to systemic (whole body)	biomarkers sometimes result from no endogenous process but rather originate from exogenous sources (e.g., naturally present in diet)
must be excreted into sewage	excretion via urine rather than feces poses fewer sampling and analytical challenges	excretion via feces creates a non-homogenous sample stream and requires more comprehensive sample preparation
confounding of biomarker levels by exogenous sources is minimal	low occurrence in raw or cooked foods (or nutritional supplements), which are often disposed directly into sewers	input possible from other animals where industrial/agricultural sewage is mixed with human sewage (can confound data)
confounding of biomarker levels by other variables is minimal	levels can be perturbed by medications	medications can up- or down-regulate biomarker biosynthesis
minimal intra-individual variance in daily excretion	daily basal levels excreted by an individual vary minimally over time (minimal diurnal or seasonal fluctuations)	excessive variation will obscure fluctuations due to changes in health
minimal inter-individual variance in daily excretion	per capita daily excretion across a population varies minimally	a wide spectrum of physiological variables can dictate the excretion of biomarkers (age, gender, genetics)
daily per capita excretion in sewage is independent of extraneous variables not considered risk factors	minimal effect from season, weather, geographic locale, medications, water-use restrictions	genetic determinants can be a function of geography
occurrence levels in sewage independent of design and usage of sewerage system	length of sewerage distribution pipes and residence time of sewage in pipes	time-dependent degradation by microorganisms during sewage transit can lead to variable reductions in biomarker levels
minimal degradation of biomarker in flowing sewage (levels persist in sewage)	slow degradation allows sampling raw sewage further downstream, permitting better mixing of influent "pulses"; ensures minimal loses during transit through sewer connections of varied lengths and residence times	sewage pulses with widely varying biomarker levels (compounded by changing pulse frequency) greatly increase the required frequency of sampling[b]
levels in raw sewage are well above method detection limits (MDL)	few analytical interferences; easier implementation of a routine method	isobaric biomarker isomers often become common interferences; this is particularly problematic, for example, with isoprostanes; many potential biomarkers with potent physiological action are not useful since they are excreted at extremely low levels

minimal potential for exogenous interference from other sources	exogenous sources include residues of target analyte on analyst's hands	residues of target analytes[b] can sometimes be excreted in sweat[c]
homogenous distribution; biomarker preferably partitions to aqueous phase	minimal partitioning to dissolved or suspended solids or sludge	partitioning to solids increases the complexity of sampling and sample preparation; this occurs especially when a biomarker is excreted via the feces
minimal degradation of biomarker in sampled sewage (levels persist in stored sewage samples)	refractory to microbial degradation or to further physicochemical degradation during sample shipment or storage	preservatives may be required to inhibit microbial degradation in stored or shipped samples
minimal de novo, ex vivo formation of analyte in sewage	minimal formation by microbial activity during sewage transit and during sewage treatment	sampling of raw sewage as early as possible in influent stream may be necessary
minimal sample clean-up and sample preparation	requires minimal pre-concentration to meet MDL	excretion of biomarker in the form of conjugates may require time-consuming hydrolysis step
analytical determination uses instrumentation routinely available; analytical methodology amenable to standardization	conventional GS/MS, LC/MS, or immunoassay	innovative "research grade" methodologies are too costly or complex for wide implementation
minimal capital investment in instrumentation; minimal analyst time	allows for high-frequency sampling	"research grade" methodologies are too costly for wide implementation
amenable to high sample through-put	amenable to automation; reduces cost	analyst intervention reduces timeliness of results
potential for in-stream continuous sampling or monitoring	Equilibrium passive samplers (EPS) allow for passive, time-integrated sampling;[d] in-stream sensors facilitate real-time data	discrete sampling gives biased results because of stream heterogeneity and sewage pulses
minimal occupational hazards for technicians	minimal hazards from samples, and from analytical reagents or reactions	handling raw sewage poses risks associated with pathogen exposure

[a] adapted in part from Daughton (2012).

[b] The challenges associated with obtaining representative samples from an STP are discussed by Ort et al. (2010).

[c] Daughton and Ruhoy (2009).

[d] examples of EPS (Zabiegała et al., 2010) include polar organic chemical integrative samplers (POCIS) and semipermeable membrane devices (SPMD).

17. Table 2. Disease or health factors that correlate with IsoP levels [a]

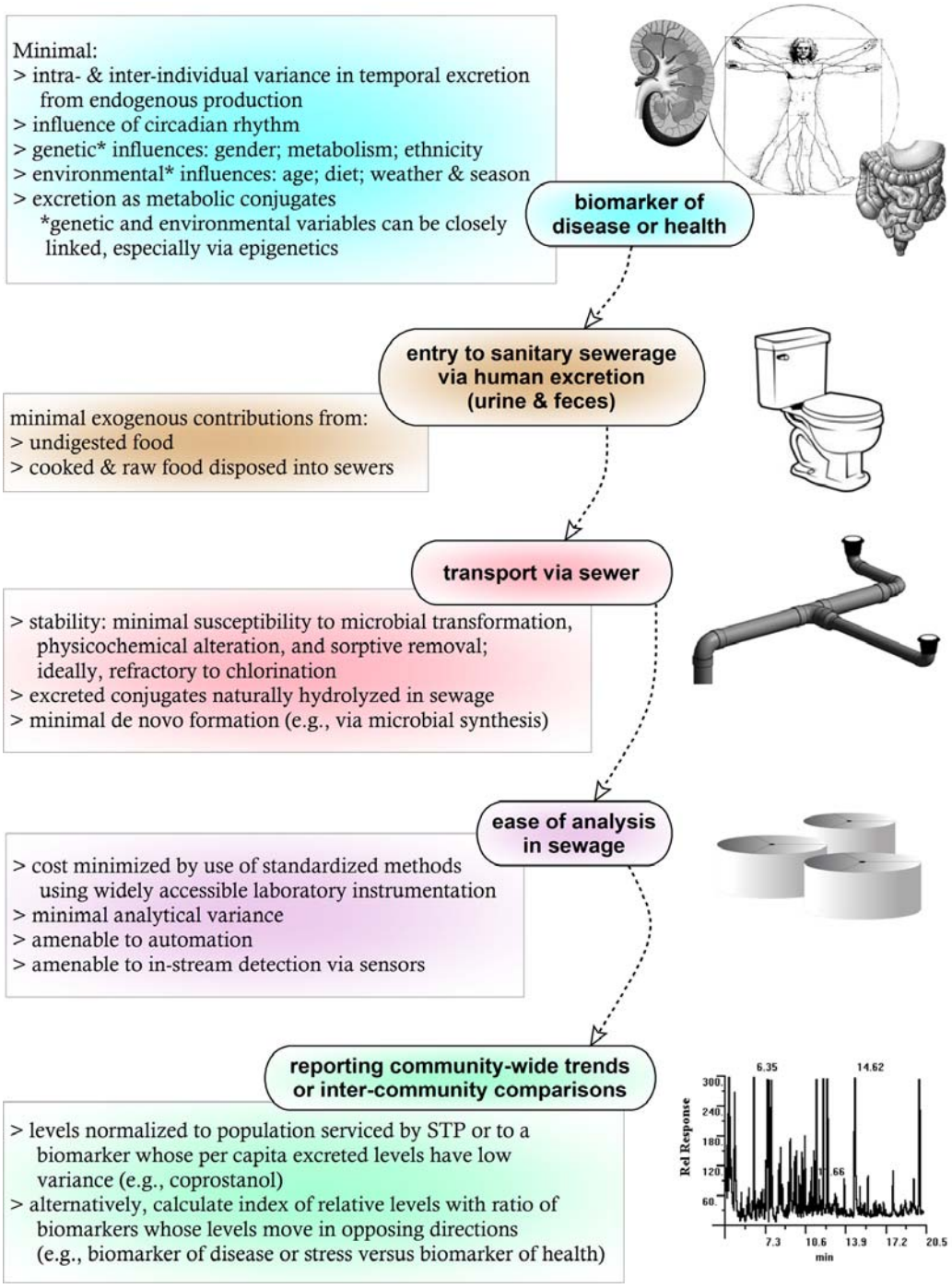
disease or health factor	relative urinary IsoP levels (versus controls)
metabolic syndrome (multiple risk factor syndrome)	
• obesity (and higher BMI and waste circumference)	Several fold to an order of magnitude higher; can be a function or baseline status; levels can reverse with dieting
• diabetes	Roughly 3-fold higher; even higher levels with glycemic excursions
• cardiovascular disease (CVD)	Elevated for most forms of CVD; also correlate with other markers of CVD and with severity
smoking	Several fold higher and strongly correlated; gender differences occur; also higher with passive smoking; sensitive to starting and stopping
pulmonary disease and asthma	Levels are often higher, but strongest correlations are in exhaled breath condensate (EBC)
psychological stress and depression	Levels seem to vary inversely with positive mood scores
cognitive decline and Alzheimer's disease	Evidence points to possible correlation
alcohol consumption	One of the strongest correlations; levels can increase many-fold; most data, however, derive from plasma
cancer	Increased levels with some cancers (e.g., liver, lung, prostate), but not all (e.g., breast)
associations with other diseases or conditions	Sometimes dramatic correlations with: rheumatoid arthritis, Lyme disease, chronic fatigue syndrome, autism, pregnancy, Dengue fever, ischemic stroke
exercise	Complex correlations (both positive and negative) depending on level and duration of exertion
drug usage correlations	Levels can be elevated or depressed during treatment with certain drugs (e.g., valproic acid) or consumption of illicit drugs; many studies show mixed correlations
associations with various non-stressor variables	
• gender	Significant gender differences are often seen but can reverse among studies
• age	Levels of parent IsoPs often increase with age (beginning at age 5)
• genetics/heritability	Environmental factors seem to have a larger influence than genetics

[a] see Supplementary materials for discussion and supporting references.

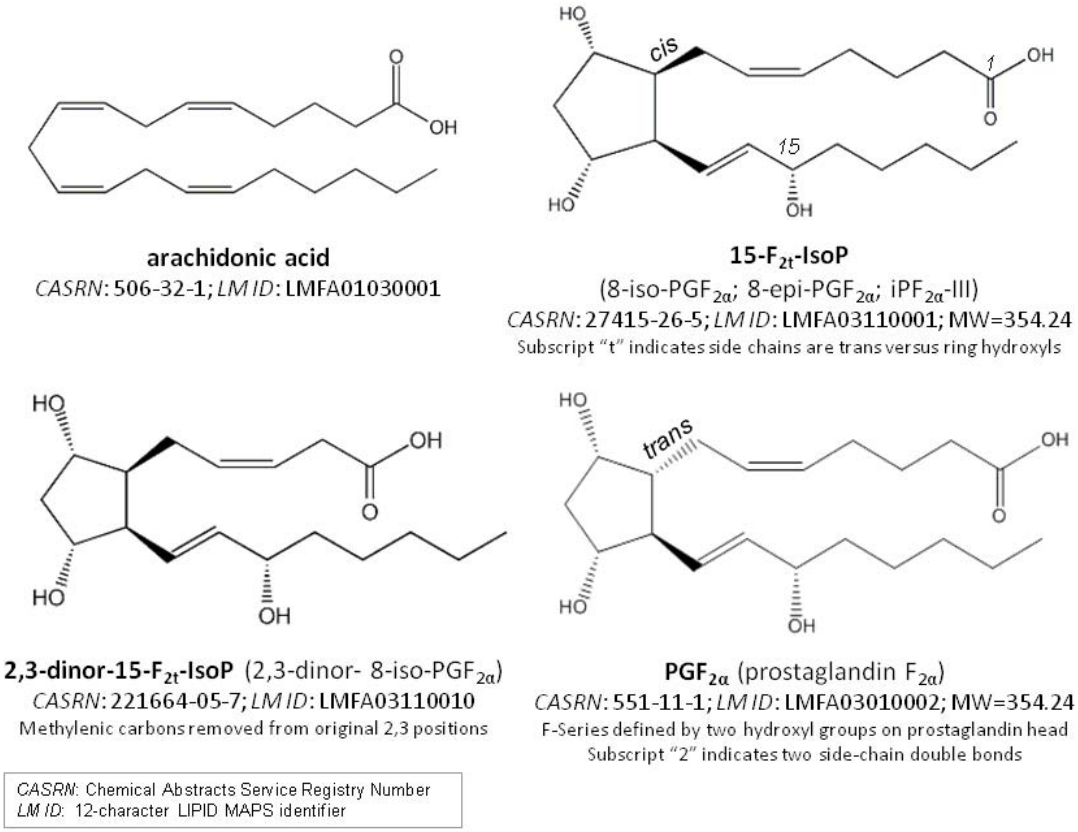
18. Table 3. Factors with potential to confound IsoP levels for use in BioSCIM [a]

confounding factor	examples
raw and cooked foods as possible exogenous source of IsoPs	Lipid oxidation during frying (e.g., foods high in polyunsaturated fatty acids); however, calculations show that this source could be a minor contributor to IsoP levels in sewage (compared with levels originating from endogenous metabolism). IsoPs seem to occur only at low levels in raw foods.
dietary lipid influence on IsoP production	Dietary omega-3 fatty acids (e.g., EPA and DHA) can lead to reductions in IsoP formation (possibly by competing with arachidonic acid during oxidative stress); in this sense, omega-3 fatty acids could be viewed as factors protective of oxidative stress. Certain unsaturated fatty acids, however, can inhibit the metabolism of IsoP, leading to enhanced levels.
exogenous antioxidants as inhibitors of IsoP production	Antioxidants in foods and in nutritional supplements can be viewed either as potential confounding factors or as a reflection of a behavior that can improve health. Studies of the influence of antioxidants on reducing IsoP levels are often inconsistent and sometimes contradictory. Dosage and overall baseline health status are determinants in outcomes.
other dietary influences on IsoPs	Caloric restriction increases IsoP excretion in obese subjects. Hyperglycemia can increase IsoP levels several fold.

[a] see Supplementary materials for detailed examples and discussion, along with supporting references.



19. Fig. 1. Characteristics of an ideal biomarker for BioSCIM



20. Fig. 2. Example of a common isoprostane (and a prostaglandin isomer), together with a dinor beta-oxidation product, and the parent arachidonic acid

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Supplementary Materials

Using biomarkers in sewage to monitor community-wide human health: Isoprostanes as conceptual prototype

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Content

1. Further rationale underlying the BioSCIM concept

An efficient, timely, and holistic approach for revealing cause-effect linkages between environmental stressors and the health of human populations persists as an unmet need. Formidable challenges are faced in developing approaches for collecting community-wide exposure and effects data suitable for assessing community-wide health. Chemical stressors have long attracted the most attention with regard to evaluating potential exposure. Chemical monitoring studies have traditionally focused on the presence of stressors in air, water, soil, biota, food, and human tissues. But data on the occurrence of chemical stressors only provides insight on the potential for exposure and reveals nothing about unknown numbers of other stressors that may well be present but which targeted chemical monitoring is unable to detect.

Little progress has been made in developing the means to measure bona fide exposure community-wide. No ability exists for measuring collective exposure to all stressors - - both chemical and non-chemical. Even less progress has been made in measurement of public health outcomes (effects) other than for infectious disease. The need for the ability to establish linkages between community-wide exposure and effects has been long recognized in the creation of state and federal projects intended to assess, track, and promote public health. This has been a focus, for example, of the Council of State and Territorial Epidemiologists (CSTE) and the Centers for Disease Control and Prevention (CDC) - - for example, through the State Environmental Health Indicators Collaborative (SEHIC) (CSTE, 2011); the use of environmental public health indicators (EPHI) was first described by the CDC/CSTE in 2000 (Malecki et al., 2007). The indicators suggested for EPHI, however, are not capable of quickly measuring population-wide exposure or systemic disease or stress.

Historically, many factors conspire as key limitations to risk assessment. Inability to assess relative cumulative and aggregate risks across all stressor classes prevents the most efficacious alignment of resources for reducing or minimizing adverse health outcomes or for promoting desired outcomes (such as improving well being). These failures can lead to incongruous investment of resources - - where overall cost-benefit remains unknown. Even if decisions to

invest resources in limiting human exposure for one class of chemicals rather than another might be based on toxicological data (albeit perhaps indirect evidence such as from animal testing), can meaningful comparisons be made among disparate stressor classes?

These limitations have led to widespread confusion among the public and regulators regarding absolute or relative risks and to continual disagreement in how to optimally allocate resources for preventing or mitigating risks, or for actively promoting health.

Stressor characterization and exposure studies play integral roles in assessing the potential for adverse human health outcomes. This approach examines the source-exposure-effects continuum unidirectionally - - forward from the source (source-based) (Daughton and Brooks, 2011; see Figure 8.1, therein). Because of the need to focus on particular individual stressors (or classes of stressors sharing common mechanisms of action), these forward-directed, source-based studies are limited by their inability to deconvolute or disaggregate the complexities of real-world exposure, a hallmark of which is cumulative exposure. Unknown numbers of physicochemical and psycho-social stressors (originating from both the natural environment and from anthropogenic sources or activities) continually act to perturb biological homeostasis from simultaneous and sequential exposures. While social and psychological stressors are traditionally studied in isolation from chemical stressors, in the final analysis all stress is ultimately mediated by way of chemical/biochemical pathways that lead to the manifestation of overt stress or disease.

The magnitude and complexity of assessing human risk from exposure to myriad numbers of chemicals (most of which are possibly unknown) is becoming more widely recognized. In March 2011, as one example, eight scientific organizations dedicated to the study of various aspects of human exposure published a joint letter offering their expertise in advancing the science behind the "exposome" (a measure of combined, total exposures experienced during an individual's lifetime) (The American Society of Human Genetics et al., 2011).

Largely absent from the practice of cumulative risk assessment have been tools for facilitating examination from the opposite direction along the continuum - - backward from effects toward

the source (effects-based). Indeed, an examination of the tools available to communities for conducting cumulative risk assessments shows an exclusive focus on exposure stressors (primarily limited to chemical and microbiological). Endogenous biomarkers indicating actual health effects have yet to be adopted for use.

Combining these two opposite approaches (source- and effects-based) could accelerate the development of more efficient and comprehensive risk assessment methodologies. An obvious means of effects-based assessment could be afforded by epidemiology - - a discipline that has received surprisingly little attention in cumulative risk assessment (Levy, 2008). The importance of an approach that works backward along the source-effects continuum has become increasingly clear as our understanding of the universe of potential stressors continually expands, serving to greatly complicate source-based approaches. Without a comprehensive, holistic understanding of the complete domain of all stressors involved with exposure, mitigation or prevention measures designed to maintain or improve public health at the community-wide level are at best only partially informed or at worst misinformed. An effects-based ("backward") approach necessarily ensures complete accommodation of the joint contributions from all pertinent stressors *(despite whether their presence is actually known*), acting along all biological pathways - - during all periods of real-world, dynamic exposure. By working from both directions at the same time, the risk assessment process could be made more efficient and holistic.

Effects-based approaches have relied on epidemiology coupled with programs such as biomonitoring (for both chemical stressors and biomarkers of stress or cellular dysfunction). This approach can provide valuable information but usually consumes significant resources and time - - as data must be derived from samples physically collected from limited numbers of individuals. Biomarkers of exposure (usually synthetic bioaccumulative environmental pollutants, such as halogenated chemicals) have traditionally been the primary focus of human biomonitoring (HBM) (CDC, 2010). HBM has relied on targeting pre-selected individuals assumed to be representative of the population under study; these studies generally aim to provide estimates of average body burdens of common pollutants.

In contrast, much less attention has been devoted to monitoring for biomarkers of effects, possibly because of the heightened analytical challenges and the unknowns surrounding the fidelity of causal connections between the putative markers and biological effects; after all, few biomarkers of effects have been sufficiently validated for wide adoption in clinical practice (Bossuyt, 2011; Ioannidis and Panagiotou, 2011).

Indeed, the National Research Council (NRC) in the 2009 report "Science and Decisions: Advancing Risk Assessment" (NRC, 2009) devoted minimal attention to the use of biomarkers in community-wide risk assessment. Such a role for biomarkers is perceived as being excessively resource intensive, primarily because the source of requisite data is presumed to originate solely from conventional HBM studies. The possibility of all-inclusive population-level "remote" monitoring (an approach that would bypass the need for directly involving individuals) had not been considered.

Real-time data for assessing health would be valuable not just to scientists and public health authorities, but also to the public. It holds potential as another tool that could help in influencing change in deleterious human behaviors. Real-time population-wide health data could facilitate a key ability never before available. It would make possible what are essentially quick or short-term experiments ("what-if" experiments) involving changes to limitless numbers of variables suspected of impacting health (beneficially or adversely). A major advantage of real-time biomarker monitoring would be the ability to detect newly emerging trends regarding overall biological stress. This could theoretically enable community-wide interventions before untoward effects become overt.

Worth noting is what will be the inevitable desire to establish cause and effect linkages between BioSCIM data and stressors thought to be acting on a community. The actual utility of BioSCIM if applied to research on causality (linking any observed or measured effects with variables purported to be causative) would be challenged by difficult questions originating with a fundamental issue in public health research - - namely, establishing causality in a system comprising profoundly complex interactions when intervention measures are instituted. This

fundamental issue certainly exceeds the scope of the discussion here, but it is the subject of substantial historic and ongoing work (Rickles, 2009).

2. Historical perspective

Sewage has rarely been considered as a resource or tool for solving problems. Although much information may have originally been associated with the individual chemical constituents of sewage, this information content is largely obscured by mixing and by myriad chemical reactions. Hidden within this litter are countless chemicals excreted after processing by human metabolic transformation effected by endogenous pathways and by the extraordinarily complex microbiome of the human gut. Reclaiming this information is an endeavor only recently contemplated (Daughton, 2012). Perhaps the first idea to emerge for mining the chemical information content of sewage was the monitoring of trace residues of illicit drugs (and their unique transformation products) for the purposes of back-calculating community-wide drug consumption (see: Daughton, 2011); the concept has since been extended to estimating the consumption of common substances such as ethanol (Reid et al., 2011). This has now opened the door for previously unforeseen and potentially revolutionary uses from mining the information associated with sewage chemical litter.

3. Lipidomics context

A very brief overview of the larger context of lipidomics in which IsoPs exist is useful in appreciating that they also serve in many respects as surrogate measures for many other oxidized phospholipids (oxylipid) biomarkers. The roles of IsoPs with respect to oxylipids are covered in the comprehensive review of Bochkov et al. (2010). The diverse roles in disease played by the many products of lipid peroxidation have been comprehensively covered in a number of reviews (e.g., Negre-Salvayre et al., 2010; Niki, 2009; Palmieri and Sblendorio, 2007; Spickett et al., 2010).

The primary eicosanoid biosynthetic pathways comprise those acting directly on the n-6 PUFA arachidonic acid (AA) by the three major families of enzymes: cyclooxygenase [COX: producing the prostaglandins and thromboxanes]; lipoxygenase [5-, 12- and 15-LOX: producing, for example, hydroxyeicosatetraenoic acids (HETE), hepoxilins (HX), lipoxins (LX), and leukotrienes (LT)]; and cytochrome P450 (CYP) epoxygenase and ω-hydroxylase families of enzymes [producing HETE and epoxyeicosatetraenoic acids (EET)]; also produced are others resulting from the non-enzymatic oxidation (such as HETE from AA). The complexity of this bare-bones framework is shown in the elegant review of Buczynski et al. (2009).

Numerous aldehydes are produced by the peroxidation of PUFAs, particularly from the omega-3 (n-3 or ω-3) and omega-6 essential fatty acids. These secondary by-products of lipid oxidation are reactive (especially with proteins) (Salomon and Gu, 2011; Salomon et al., 2011) and are probably involved in myriad reactions with endogenous biochemicals, possibly playing central roles in diseases, such as diabetes. Two common examples are 4-hydroxy-2-nonenal (HNE), which results from peroxidation of omega-6 fatty acids (mainly linoleic and arachidonic acids), and 4-hydroxy-2-hexenal (HHE), which results from peroxidation of omega-3 fatty acids (e.g., EPA and DHA, which differ by the position of a fatty acyl double bond) (Guéraud et al., 2010). HHE and HNE, for example, have been shown to form covalent adducts with certain histidine groups in the beta-chain of human insulin. By modifying the structural conformation of insulin, alkenals may thereby reduce the potency of insulin - - disrupting its storage, secretion, and potency, and thereby possibly contributing to insulin resistance (Pillon et al., 2011). Their reactivity, however, limits their potential usefulness as excreted biomarkers detectable in sewage.

4. Aspects of biomarkers not suitable for BioSCIM: an example of DHEA

A number of other factors that can exclude a biomarker from use with BioSCIM are illustrated by the adrenal androgen dehydroepiandrosterone (DHEA). Endogenous DHEA (and its sulfoconjugate DHEA-S) is the most abundant circulating adrenal steroid and plays a significant role as precursor to the sex steroids and as an antagonist for cortisol. DHEA is one of the most frequently included biomarkers in clinical studies of allostatic load – and one of only two markers purported to track positive health (high-density lipoprotein [HDL] being the other)

(Piazza et al., 2010); see section **Error! Reference source not found.**: Biomarker Profiles and Community-Wide Allostasis.

Although other natural contributory sources of DHEA into sewage are perhaps rare, since DHEA biosynthesis appears to be limited to humans and certain other primates (Nguyen and Conley, 2008), its presence in sewage could be confounded by other exogenous sources - - one being its growing use as a nutritional supplement and sometimes as an abused drugs in sports (Mareck et al., 2007). Although DHEA is excreted in comparatively large amounts, it is also subject to large inter- and intra-individual variation in daily excretion rate (variations of an order of magnitude and more) (Pfeifer and Spiteller, 1981), and its overproduction can result in certain diseased states. Its endogenous levels are also affected by a variety of drugs (Salek et al., 2002). DHEA's excretion rate strongly correlates with age, with levels dropping about 2% per year after the 20s and varying by nearly 3 orders of magnitude in those under 20 (Remer et al., 2005).

The stability of DHEA in sewage could also be a factor, as it readily reacts with hypochlorous acid (the reactivity of its sulfoconjugate is unknown) (Mash et al., 2010). One study showed that at least for the effluent from two STPs, DHEA was present only at low ng/L levels - - and sometimes not at all (Vulliet et al., 2007). DHEA has rarely been targeted for monitoring in the environment; one of the few studies targeted DHEA-S (Yamamoto et al., 2006). A rare study of its occurrence in raw sewage (from 12 STPs) found a somewhat broad range, from 0.58-2.17 µg/L (mean of 1.39) (Tabak et al., 1981).

5. Factors and variables affecting regulation of IsoP production: disease, lifestyle, and genetics

5.1. Metabolic syndrome

The associations of IsoP with metabolic syndrome (also known as multiple risk factor syndrome) are strong and highly interdependent. Metabolic syndrome comprises multiple co-morbidities, all of which act together in increasing the risk of developing cardiovascular pathologies and diabetes. Obesity (and associated measures such as BMI and waist circumference) is one of the primary initial symptoms and causes. Metabolic syndrome is a complex aspect of oxidative

stress, which seems to play integral roles in its development. Only a few representative studies can be highlighted here. Most studies examine multiple co-morbidities, so it can be difficult to discuss the associations in isolation from each other.

5.1.1. Obesity

The role of oxidative stress in human obesity has been reviewed (Vincent et al., 2007; Vincent and Taylor, 2006) as has its role in childhood obesity (Ostrow et al., 2011). In general, urinary IsoP levels range from several fold to an order of magnitude higher in obese individuals.

For 2,828 subjects from the Framingham Heart Study, urinary 8-epi-$PGF_{2\alpha}$ was found to most strongly correlate with smoking, diabetes, and body mass index (Keaney et al., 2003). Plasma 8-epi-$PGF_{2\alpha}$ levels in children increased multi-fold with severity of obesity and number of complications (Araki et al., 2010). Urinary IsoPs were found to be strongly correlated with the degree of obesity in children (Savino et al., 2011). In a study of 42 obese pre-teen children and 34 non-obese children, urinary IsoP was associated with obesity and early abnormalities in blood pressure. Mean urinary IsoP in obese subjects was 29.7±30.7 versus 18.2±10.6 pg/mg creatinine for non-obese subjects. For those in the 95^{th} percentile level for blood pressure versus those below the 95^{th} percentile level, urinary IsoP was about 3-fold higher: 79.8±65 vs. 23.5±23.7 pg/mg creatinine (Ostrow et al., 2011). In the IRAS cohort, four urinary F_2-IsoPs (especially 2,3-dinor-iPF(2_α)-III) were also associated with obesity (Il'yasova et al., 2011); in this study, however, urinary F_2-IsoP levels were found to be inversely associated with subsequent weight gain - - presumably reflecting higher overall rates of metabolic fat oxidation.

Urinary 8-epi-$PGF_{2\alpha}$ was significantly correlated with BMI and waist circumference (Furukawa et al., 2004; Mori et al., 1999). In a weight-loss study of 36 healthy but obese women and 30 healthy normal-weight women, plasma 8-iso-PGF_2 was followed along with other markers such as CRP and the cytokine interleukin-6 (IL-6). Prior to weight loss, IsoP was more than 10-fold higher in the obese group. After weight loss (ca 20%), the levels of IsoP for the obese group were reduced to less than one-third their initial levels (Bougoulia et al., 2006). By inducing an increase in non-esterified fatty acids, excretion of F_2-IsoPs were found to increase in obese hypertensive patients compared with healthy controls (Stojiljkovic et al., 2002).

In perhaps the first study on the effect of weight loss on urinary IsoP, 19 subjects with untreated metabolic syndrome were placed on a weight reduction program (12-week calorie restriction, which resulted in a final but modest weight loss of 4-5%), and their urinary IsoP was compared with 18 subjects who maintained their weight. There was no effect of weight loss on IsoP excretion. For those maintaining their weight, the excretion of IsoP (pmol/24 h) was 10.0 (8.41-11.88) [baseline] and 8.95 (7.43-10.79) [conclusion] and for those who lost weight the levels were 9.02 (7.59-10.69) [baseline] and 9.04 (7.50-10.89) [conclusion] pmol/24 h (Tsai et al., 2009).

A cohort of 845 women who were free of cancer diagnosis, serving as controls in the Shanghai Women's Health Study (SWHS), served as one of the first studies correlating urinary F_2-IsoPs and 15-F_{2t}-IsoP-M (2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP or 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$) with BMI (and age). This study provides a large body of data (Dorjgochoo et al., 2011). A prospective study nested within the SWHS (nearly 75,000 women) on correlations among oxidative stress, obesity, and breast cancer risk, yielded some paradoxical findings. Urine was analyzed for 15-F_{2t}-IsoP and its metabolite 15-F_{2t}-IsoP-M. Urinary excretion of IsoPs was found to not be significantly different between those women later developing breast cancer and controls. But the data for the two sub-groups contrasted dramatically. For those women who were overweight (BMI>25), increasing excretion of IsoPs was positively correlated with increased breast cancer risk (and this correlation grew stronger with increasing BMI). But for non-overweight women, reduced excretion of IsoPs correlated with increased breast cancer risk. The study found that the correlation of oxidative stress with development of breast cancer changes from positive to negative as the BMI is reduced (Dai et al., 2009; Dai and Zhu, 2009).

5.1.2. Diabetes

In general, urinary IsoP levels are up to roughly 3-fold higher in diabetics than in healthy subjects, but substantially higher levels can result from glycemic excursions. A comprehensive review of the role played by IsoPs in diabetes covers the pre-2000 literature (Mezzetti et al., 2000). An early study showed an association between plasma 8-epi-$PGF_{2\alpha}$ and non-insulin dependent diabetes mellitus (Gopaul et al., 1995).

A comprehensive discussion of the role of plasma glucose and its fluctuating levels on lipid oxidation is available (Monnier et al., 2006). Mean urinary 8-isoPGF$_2$ was significantly higher in 21 patients with type 2 diabetes than in controls (482±206 versus 275±85 pg/mg creatinine). The highest levels resulted from glycemic excursions. These spikes varied by 5 fold, up to about 1,000 pg/mg creatinine (Monnier et al., 2006). In 21 type 2 diabetics, plasma F_2-IsoP increased 34% during induced acute hyperglycemia (Sampson et al., 2002). Urinary 8-iso-PGF$_{2\alpha}$ was roughly 2-fold higher with both forms of diabetes than in healthy controls (400 versus 200 pg/mg creatinine). Improving metabolic control in those with poorer glycemic control led to corresponding and linear reductions in 8-iso-PGF$_{2\alpha}$ (Davi et al., 1999).

In 28 patients with type 2 diabetes, urinary F_2-IsoP was 720.37±454.89 pg/mg creatinine versus 375.08±440.64 pg/mg creatinine for controls. Noteworthy, however, was that for 8 of the 19 control subjects, urinary IsoP was below the limit of quantitation (200 pg/mL) (Nartnampong et al., 2008).

In 50 type 2 diabetics, urinary F_2-IsoP was roughly 3-fold higher than in 25 controls (Devaraj et al., 2001). The highest urinary IsoP levels were associated with those patients receiving the highest and lowest insulin doses, showing a hormetic relationship (Monnier et al., 2011). With 14 type 1 diabetics, urinary 8-epi-PGF$_{2\alpha}$ was reduced upon insulin treatment (from 92.0±41.5 to 66.9±28.9 pg/mg urinary creatinine) compared with controls (39.1±13.8 pg/mg creatinine) (Flores et al., 2004). With the IRAS cohort of non-diabetics, higher levels of urinary F_2-IsoPs were correlated with lower risk of diabetes - - presumably reflecting higher rates of fat oxidation (Il'yasova et al., 2011).

5.1.3. Cardiovascular disease

The literature involving IsoPs and coronary diseases involves cardiovascular disease (CVD), coronary heart disease (CHD), coronary artery disease (CAD), peripheral arterial disease (PAD), congestive heart failure (CHF), atherosclerosis, and myocardial ischemia, among others. This literature also involves allied risk factors such as hypertension, hyperlipidemia, blood pressure, and other markers such as C-reactive protein and various measures of cholesterol such as LDL.

For example, elevated IsoP levels correlate with higher levels of oxidized low-density lipoproteins (oxLDL) and lower levels of high-density lipoproteins (HDL) (Kennedy et al., 2005). This finding is interesting given that F_2-IsoPs physically associated with HDL are several fold higher than in LDL or VLDL lipoproteins. HDL may therefore be serving in a protective role by scavenging IsoPs (Proudfoot et al., 2009). The inhibition of angiogenesis might serve as a common mode of action by which IsoPs contribute to coronary diseases (Benndorf et al., 2008; Gnann, 2009); perturbation of vasomotor function is probably another pathway (Hou et al., 2004).

A review of the roles played by IsoPs in initiation, perpetuation, and progression of atherogenesis is presented by Praticò (2008). A review of the pre-2005 literature surrounding IsoP production and CVD is presented by Patrono et al. (2005). Other overviews are available (e.g., Cracowski and Durand, 2006; Schwedhelm et al., 2004; Schwedhelm and Böger, 2003; Young, 2005).

A few representative studies include the following. In 100 patients with CAD, spot urinary 8-IsoP was highly correlated with the severity of the disease. Average levels were 69 pg/mL versus 38 pg/mL for patients without CAD; 8-IsoP levels also correlated with other markers such as C-reactive protein (Basarici et al., 2008). In a study of 50 subjects with CAD and 54 controls, plasma F_2-IsoP was a strong predictor of CAD (Shishehbor et al., 2006). Urinary F_2-IsoPs were strongly associated in postmenopausal women with increased incidence of fatal CVD (Roest et al., 2008). In patients hospitalized with severe CHF, urinary 15-F_{2t}-IsoP concentrations might represent instances of maximally increased levels: 600 (355-720) pg/mg creatinine upon admission versus 280 (148-425) pg/mg creatinine for those admitted with mild CHF or 198 (125-281) pg/mg creatinine for control subjects (Nonaka-Sarukawa et al., 2003). In a study of 15 atherosclerotic patients with carotid artery sclerosis, urinary 8-iso-$PGF_{2\alpha}$ was roughly in the range of 175-350 pg/mg creatinine versus 150-200 pg/mg creatinine for controls (Cavalca et al., 2010).

5.2. Smoking

Substantial published data generally correlates increased urinary levels for most (but not all) IsoPs and IsoP metabolites upon exposure to both active and passive cigarette smoke; urinary levels of F_2-IsoPs are frequently elevated by up to 2 fold but sometimes higher. Morrow's group was perhaps the first to show increased in vivo oxidative injury in active smokers assessed via 8-epi-$PGF_{2\alpha}$; but levels were not elevated in all smokers, and levels also did not correlate with the quantity or duration of smoking, or with cigarette tar and nicotine content (Morrow et al., 1995).

Urinary 8-epi-$PGF_{2\alpha}$ in human smokers was roughly 2-fold higher than for non-smokers, with relatively low inter-individual variation across days (Bachi et al., 1996). Urinary 15-F_{2t}-IsoP was 40% higher in 33 healthy smokers than in 58 age-matched controls (1.41 versus 1.01 ng/mg creatinine) (Campos et al., 2011). In a study of 677 apparently healthy Japanese, mean urinary concentration of 8-IsoP was increased significantly by smoking, with frequency of smoking being the strongest determinant (Sakano et al., 2009b); another study of 551 apparently healthy Japanese subjects also showed a strong association of urinary 8-IsoP with smoking (Dohi et al., 2007).

A study of 67 healthy smokers and 65 non-smokers showed roughly twice the level of urinary 8-epi-$PGF_{2\alpha}$ in smokers (normalized to creatinine for 24-hour samples) (Zedler et al., 2006). Urinary 2,3-dinor-8-iso-PGF_2 was also roughly 2-fold higher among smokers (Liang et al., 2003).

A study of 178 otherwise healthy subjects (119 smokers and 59 controls) was the first to examine a comprehensive spectrum of biomarkers of oxidative damage in both plasma and urine. Basal levels of urinary F_2-IsoPs were more than 4-fold higher among chronic smokers versus non-smoker controls. Upon 1 hour after smoking a single cigarette, the levels were more than 6-fold higher. Although the basal levels of the dihydro and reduced dinor metabolites (2,3-dinor-5,6-dihydro F_2-IsoPs and 2,3-dinor F_2-IsoPs) were the same for smokers and controls, the levels of the two metabolites increased after smoking. These data show that the urinary levels of the dinor metabolites relative to the parent F_2-IsoP can vary dramatically and are not necessarily highly correlated for smokers (Seet et al., 2011).

In a large study of 3,585 adult smokers and 1,077 non-smokers, urinary 8-epi-$PGF_{2\alpha}$ was monitored as one of 29 biomarkers. Of all 29 biomarkers, 8-epi-$PGF_{2\alpha}$ was the most strongly correlated with smoking - - with its mean 24-h excretion being 42% higher among smokers compared with non-smokers (1,890 ng/24h versus 1,331 ng/24h) (Frost-Pineda et al., 2011). BMI and age both had strong influences. In the 1,610 participants (multi-race/ethnic sample of midlife women) of the Study of Women's Health Across the Nation (SWAN), the median urinary F_2-IsoP concentration among smokers was more than twice that of non-smokers: 917 ng/L (inter-quartile range [IQR]: 467, 1832 ng/L) versus nonsmokers: 403 ng/L (IQR: 228, 709 ng/L) (Tomey et al., 2007). For certain isomers (e.g., 8,12-iso-PGF_2 -VI), however, urinary levels in smokers can approach an order of magnitude higher than in non-smokers (1.9 ng/mg creatinine versus 0.2 ng/mg creatinine) (Mesaros et al., 2009).

Studies have not provided uniform correlations, however. One problem might pertain to all correlation studies - - namely, the measurement basis upon which data are expressed. One example is shown in the comprehensive study of the 24-h excretion of $iPF_{2\alpha}$-III, 15-epi-$iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, 8,12-iso-$iPF_{2\alpha}$-VI, and 2,3-dinor-$iPF_{2\alpha}$-III. Interpretation of the data depended on how the data were expressed. For example, clear differences between levels excreted daily from smokers and nonsmokers were shown for both $iPF_{2\alpha}$-III (0.592±0.246 vs. 0.399±0.109 μg/day) and its metabolite 2,3-dinor-$iPF_{2\alpha}$-III (3.537±1.346 vs. 2.723±0.768 μg/day). But when the same data were expressed in terms of urinary concentration (pg/mL), the difference between smokers and nonsmokers disappeared for 2,3-dinor-$iPF_{2\alpha}$-III. For 8,12-iso-$iPF_{2\alpha}$-VI, its levels were not significantly different among the two groups regardless of the three bases of measurement for expressing excretion (Yan et al., 2007).

A comprehensive examination of urine from healthy, college-aged nonsmokers (n=6M/8F) and smokers (n=6M/5F) revealed gender differences for a variety of IsoP isomers, with women having higher F_2-IsoP metabolite levels than men (Taylor et al., 2008).

Although 8-epi-$PGF_{2\alpha}$ has been established as resulting from oxidation injury in smokers, it was recently shown to also indicate oxidation in passive smokers (children and adults). Plasma levels

of 8-epi-$PGF_{2\alpha}$ also increased at a faster relative rate (30% per day) in passive smokers than in active smokers (15% per day) upon recurring 60-min daily exposures. Levels would also drop quickly before the next day's exposure but not return to their prior starting level (Ahmadzadehfar et al., 2006).

The increase (and decrease) of urinary 8-epi-$PGF_{2\alpha}$ is sensitive to restarting (and quitting) smoking (Chehne et al., 2001; Reilly et al., 1996). In 47 adults with pre-existing risk factors, including smoking, base levels of 8-epi-$PGF_{2\alpha}$ differed but immediately began to drop upon cessation of smoking - - especially urinary levels. The levels in all individuals, regardless of initial levels, dropped about the same (by one third) after 2 weeks of cessation - - nearly to the levels seen in healthy subjects (Pilz et al., 2000). In another study, however, former smokers still had elevated 8-iso-PGF_2 levels: median (pmol/mmol creatinine) of 184 for former smokers, 191 for current smokers, and 167 for non-smokers (Helmersson et al., 2005b); similar findings of hysteresis - - where levels in ex-smokers do not return to the levels of non-smokers - - have been reported by others (e.g., Obata et al., 2000).

In the 401 control subjects of the Long Island Breast Cancer Study Project, 15-F_{2t}-IsoP levels were higher for smokers and were also unaffected by other usual modifiers, such as exercise, intake of anti-oxidants via fruits and vegetables, consumption of alcohol intake, BMI, or menopausal status (Rossner et al., 2006).

5.3. Pulmonary disease - - asthma

There has been surprisingly little research on biomarkers of oxidative stress resulting from air pollution - - and almost none using urinalysis (Delfino et al., 2011). IsoPs are involved in the progression of a wide array of pulmonary pathologies (Janssen, 2008). In reviews of roles played by IsoPs as biomarkers of both disease activity and mediators of disease progression, clear associations are evident between IsoPs and asthma (Voynow and Kummarapurugu, 2011; Wood et al., 2003). The major urinary metabolite of 15-F_{2t}-IsoP - - 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (15-F_{2t}-IsoP-M) - - is increased roughly 25-40% (in the range of 0.4 ng/mg creatinine) within 2 hours of pulmonary challenge with an allergen (Dworski et al., 2001). In the exhaled breath condensate (EBC) from patients with mild asthma, 8-iso-$PGF_{2\alpha}$ was more than 4-fold higher than

in healthy control subjects (Zhao et al., 2008). IsoP levels in EBCs can be over an order of magnitude higher for those with a variety of lung diseases, including asthma (Janicka et al., 2010). IsoPs are also elevated in the EBC from cystic fibrosis cases (Montuschi et al., 2000). But some studies showing IsoP elevation in ECB among asthmatic children fail to show elevated urinary levels (Carraro et al., 2010). Urinary IsoP is elevated in patients with asbestos or silica-induced lung diseases (Syslová et al., 2009).

5.4. Psychological stress & depression

Assessing risk from non-chemical stressors, such as psychological stress, has always proved challenging. Non-chemical stressors, however, are deemed particularly important with respect to health disparities (Sexton and Linder, 2011). While social and psychological stressors are traditionally studied separately from chemical stressors, in the final analysis all stress is ultimately mediated by way of chemical/biochemical pathways that lead to the manifestation of overt stress or disease. Despite the fact that psychological stress is a critical component of overall systemic stress, it has received little attention in its measurement via biomarkers. An overview of psychological stress and its effects on the production of biomarkers is provided by Djuric (2008); none of these biomarkers, however, was deemed useful for BioSCIM monitoring when evaluated against the criteria in Table 1.

Oxidative stress is associated with depression (Dimopoulos et al., 2008; Maes et al., 2011a; Maes et al., 2011b). Markers of oxidative stress may reflect the quality of romantic relationships for men (Merriman, 2010). Urinary F_2-IsoPs were found to vary inversely with respect to positive mood scores among a small group of 8-10 year olds (Nasca et al., 2010). A patent claims that IsoP excretion increases under psychological stress (Cobain et al., 2004).

5.5. Cognitive decline & Alzheimer's disease

Positive correlations have been reported for urinary IsoP levels with various measures of cognitive decline (Insel et al., 2011). IsoPs are also believed to play a role in Alzheimer's disease (Praticò, 2010). But most studies have revealed only weak associations for plasma or urinary levels of IsoPs with Parkinson's disease (Connolly et al., 2008) or with Alzheimer's disease (Bohnstedt et al., 2003; Kim et al., 2004; Mufson and Leurgans, 2010).

5.6. Alcohol consumption

As mentioned earlier, some of the largest increases in urinary IsoP levels result from alcohol consumption - - especially chronic intake coupled with liver disease. For healthy adults, urinary excretion of $iPF_{2\alpha}$-III has been shown to increase about 8-fold from baseline; higher increases (e.g., 2-fold) were shown with concomitant liver disease (Meagher et al., 1999). Urinary F_2-IsoP in 16 healthy men has been shown to decrease after alcohol consumption had been reduced but not eliminated (616±73 pmol/mmol creatinine versus 503±94 pmol/mmol creatinine) (Barden et al., 2007). A preponderance of clinical studies on alcohol exposure, however, have measured IsoP levels in plasma; these have been excluded from the brief summary here.

5.7. Cancer

Chronic inflammation can play roles in all phases of carcinogenesis. Increased urinary levels of IsoPs have been associated with an increased risk for a variety of cancers. Elevated urinary IsoP correlates with increased risk of hepatocellular carcinoma (Wu et al., 2008), lung cancer (Epplein et al., 2009), and prostate cancer (Barocas et al., 2011). But in the Long Island Breast Cancer Study Project (400 breast cancer patients and 401 control subjects), only a "modest positive association" was found for urinary levels of 15-F_{2t}-IsoP (Rossner et al., 2006). Recent work has begun to synthesize an integrated picture of the linkages between oxidative stress and cancer initiation and perpetuation (Martinez-Outschoorn et al., 2010).

5.8. Associations with other diseases or conditions

IsoPs may play roles as indicators or mediators of a wide spectrum of other diseases and conditions. Urinary F_2-IsoP is significantly increased in subjects with rheumatoid arthritis (Rho et al., 2010). Lyme disease dramatically elevates (by nearly 9-fold) urinary levels of 8-isoP$GF_{2\alpha}$ (328 pg/mg creatinine versus baseline levels of 37 pg/mg creatinine) (Łuczaj et al., 2011).

Significantly elevated plasma 8-iso-$F_{2\alpha}$-IsoP (up to 2-fold) was noted in patients with chronic fatigue syndrome (CFS); those reporting the most joint pain and level of postexertional malaise had the highest IsoP levels (660 pg/mL and 492 pg/mL, respectively, compared with 319 pg/mL for controls) (Kennedy et al., 2005). Subjects with CFS had consistently higher blood levels of F_2-IsoP than healthy controls after exercise to exhaustion (Robinson et al., 2010).

Plasma F_2-IsoP significantly increased in subjects with late-stage renal disease when administered ramipril (an angiotensin-converting enzyme inhibitor) or valsartan (an angiotensin-receptor blocker) (Gamboa et al., 2012).

Urinary 8-iso-$PGF_{2\alpha}$ was significantly increased in 33 autistic children versus controls (in some it was markedly increased) (Ming et al., 2005). In a study of 26 untreated autistic children and 12 healthy controls, isoprostane $F_{2\alpha}$ -VI levels were higher in autistic subjects: 5.2±0.5 ng/mg creatinine versus 3.1±0.3 ng/mg creatinine (Yao et al., 2006).

Upon UV irradiation, 8-IsoP is produced in the skin. In a study of dermal exposure to simulated sunlight, IsoP was elevated in skin suction-blister fluid after exposure to sustained UV at the threshold dose producing sunburn (Kuhn et al., 2006). 8-IsoP was found to be a dose-related biomarker for photo-oxidative ultraviolet B damage in human skin (Schneider et al., 2006). It is unknown whether this source could lead to IsoP excretion via sweat.

Pregnancy is associated with higher levels of urinary IsoPs (0.45 nmol/nmol creatinine versus 0.24 nmol/nmol in non-pregnant women) (Ishihara et al., 2004). During pregnancy, the risk for pre-eclampsia and pre-term delivery was 5-fold higher in the highest versus lowest quintile levels of urinary IsoP (Stein et al., 2008).

Urinary IsoPs were significantly elevated across all groups at the onset and during recovery for acute morbidities such as Dengue fever and ischemic stroke but not for the chronic morbidity of Parkinson's disease. Urinary levels compared with healthy controls increased from 2- to 4-fold (4 ng/mg creatinine versus 1 ng/mg creatinine) (Lee et al., 2009).

5.9. Exercise

The associations of IsoPs and exercise are complex and often seemingly contradictory; urinary IsoP levels have been found to increase, decrease, and remain unchanged - - largely as a function of the exercise type (resistance versus endurance; anaerobic versus aerobic), intensity (sustained versus acute), volume, and duration, coupled with the subject’s systemic anti-oxidant status and overall health (Fisher-Wellman and Bloomer, 2009; Nasca et al., 2010).

Regardless of the response, IsoP levels tend to return to baseline within 1 to 24 h post-exercise. In contrast to excretion resulting from chronic diseased states, the effects of exercise on sewage levels would therefore be short-term and episodic. Research on IsoPs and exercise often involves the effect of antioxidant supplementation, which has also been found to have seemingly conflicting results - - with urinary IsoP levels increasing, decreasing, or remaining unchanged, probably partly as a function of dose (Nikolaidis et al., 2011).

As with other aspects of IsoP production, the initial baseline conditioning of the study subjects may dictate whether further reductions in urinary IsoP are even possible (e.g., Sakano et al., 2009a). When gains in aerobic fitness during a study are large, urinary IsoP levels have been observed to be reduced (Campbell et al., 2010). Likewise, aerobic exercise reduced urinary F_2-IsoP by 34% in 15 usually sedentary premenopausal women (Schmitz et al., 2008). But urinary IsoPs increased significantly in patients with idiopathic pulmonary fibrosis (IPF) after low-level exercise (Jackson et al., 2010).

Comprehensive overviews of IsoPs and exercise are available (e.g., Fisher-Wellman and Bloomer, 2009; Nikolaidis et al., 2011). Prostanoid levels in urine have recently been shown to increase far more than IsoPs - - up to 55-fold - - after exercise (Blatnik and Steenwyk, 2010). Studies involving children are just beginning (Nasca et al., 2010). Much of the IsoP data from exercise studies involves plasma levels and is largely excluded from the brief summary here.

Urinary IsoP levels correlate with the load intensity of physical exercise, with maximal production occurring upon over-training (7-fold increases) (Margonis et al., 2007). In a strenuous 50-km run, urinary 15-F_{2t}-IsoP levels increased nearly 6-fold (from 1 ng/mL to about 6 ng/mL) (Díaz-Castro et al., 2011)

Anti-oxidants can serve as prooxidants during exercise. Supplementation for 2-months with alpha-tocopherol prior to the Iron Man Triathlon served to increase plasma F_2-IsoP levels post-race in 38 triathletes by nearly 2-fold; exercise alone also increased levels 2-fold versus baseline (McAnulty et al., 2005). Similar results have been obtained for vitamin C with marathon runners

(Nieman et al., 2002). With supplementation of EPA and DHA prior to intense, sustained exercise may also significantly increase IsoPs (McAnulty et al., 2010). In contrast, a study involving supplementation with vitamins C and E found no change in urinary F_2-IsoPs after rigorous aerobic exercise (Bailey et al., 2010).

In a study of exercise with 10 healthy males, no significant difference in total F_2-IsoPs could be found up to 24 h after anaerobic or aerobic exercise (1116.4±355.7 ng/day and 1022.8±173.3 ng/day, respectively) (Shi et al., 2007). It is generally accepted that no clear trends have yet to emerge from studies regarding the effects of either acute or chronic exercise on urinary F_2-IsoPs (Nikolaidis et al., 2011).

5.10. Drug usage correlations

IsoP levels can be elevated during treatment with a wide array of medications and illicit drugs. The following summarize some of the drugs that affect IsoP production.

Epileptic children receiving valproic acid had elevated urinary levels of 15-F_{2t}-IsoP (Michoulas et al., 2006). The effect of therapy with statins (inhibitors of 3-hydroxy-3-methyglutaryl-coenzyme A reductase) on IsoP production is equivocal - - some studies reporting no change (Kom et al., 2007; Ky et al., 2008), while others reporting mild reductions (De Caterina et al., 2002; Lee et al., 2002).

Acetaminophen breaks the heme redox cycle and therefore has the potential to significantly lower IsoP production (by reducing the level of free iron serving as a catalytic oxidant). This is seen with severe muscle injury (rhabdomyolysis), where heme is released from myoglobin and hemoglobin, which in turn enhances the production of IsoPs via catalysis (Boutaud et al., 2010). Indeed, free iron may play a major role in many aspects of oxidative stress, including production of IsoPs (Kell, 2009).

Given that IsoPs are not produced via the COX pathway, non-steroidal anti-inflammatories (NSAIDs) might at first not be expected to have significant effects on IsoP production. A trial enlisting healthy patients over 70 years old but with a family history of Alzheimer's disease (AD)

studied whether naproxen or celecoxib or aspirin might prevent AD. No effect was found on either plasma or urinary F_2-IsoP (Montine et al., 2010). Likewise, high doses of aspirin (2,500 mg/day) or ibuprofen (3,200 mg/day) did not change the urinary levels of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (Morales et al., 2001).

But another perspective poses an indirect mechanism that could be facilitated by the increased availability of arachidonate via the inhibition of prostaglandin biosynthesis. Prostaglandin production can be inhibited not just by drug-based COX inhibitors (such as NSAIDs), but evidence is growing that COX can also be inhibited by myriad other chemicals, many of which are endocrine disruptors (Kristensen et al., 2010).

The effects of NSAIDs on IsoPs are sometimes an aspect of studies focused on exercise. Administration of ibuprofen before extreme exercise (an extremely difficult 100-mile ultramarathon) resulted in increased plasma and urinary F_2-IsoPs (McAnulty et al., 2007). In 29 ibuprofen users (versus the control of 25 non-user competitors), post-race urinary levels increased over 2-fold in users and none in non-users (from about 1.7 pg/mL to ca 4 pg/mL); but the authors hypothesized that IsoPs increased because of kidney damage caused by ibuprofen.

A comprehensive examination of the role of insulin in oxidative stress is presented by Monnier et al. (2010). Insulin exerts both antioxidant and anti-inflammatory activities. Urinary iso-$PGF_{2\alpha}$ was much higher in type 2 diabetics treated with oral hypoglycemic agents such as metformin, sulfonylureas, or thiazolidinedione than in diabetic patients treated with insulin (112 pmol/mmol creatinine vs. 69 pmol/mmol creatinine) or in non-diabetic controls (65 pmol/mmol creatinine); but another study showed that metformin reduced plasma 8-iso$PGF_{2\alpha}$ levels (25 pg/mL) more than sulfonylureas (31 pg/mL) in type 2 diabetics, but still not to the level of controls (17 pg/mL) (Faure et al., 2008b).

5.11. Non-stressor variables (influence of personal characteristics)

There are three primary variables that contribute to IsoP excretion variability but which are not stressors per se: gender, age, and heritable factors such as various aspects of IsoP metabolism.

5.11.1. Gender

Gender probably plays a role in determining IsoP excretion rates but the relative trends for males and females seem variable; sometimes these trends vary as a function of the parent IsoP versus the metabolites. Men are sometimes found to excrete significantly more free and total (free plus conjugated) IsoP than women. Baseline urinary 8-iso-PGF_2 levels in 52 young men and 51 age-matched women were 292±56 versus 164±25 pg/mg creatinine, respectively; in a 4-week study involving supplementation with antioxidant vitamins, urinary 8-iso-PGF_2 was reduced more in men (total reduction of 48%), possibly because of their heightened baseline level of oxidative stress resulting from metabolism (Ide et al., 2002). Urinary total IsoP excretion rates for men and women were roughly 8.7 and 6 nmol/24 h, respectively (Yan et al., 2010). In 323 apparently healthy Japanese, mean urinary 8-IsoP was significantly higher in males than females (Sakano et al., 2009a).

But other studies report higher urinary IsoP levels for women than men. One example came from the Framingham Heart Study, where urinary 8-epi-$PGF_{2\alpha}$ levels in 2,828 subjects was 16% higher in women (Keaney et al., 2003). A study of healthy, college-aged nonsmokers and smokers revealed gender differences for a variety of urinary IsoP isomers, with women having higher F_2-IsoP metabolite levels than men (Taylor et al., 2008). The mean urinary level in females of the IsoP metabolite 2,3-dinor-i$PF_{2\alpha}$-III (5.0±0.6 μg/g creatinine) was significantly higher than for males (3.6±0.3 μg/g creatinine) (Zhang et al., 2010); and another study also found higher levels of the dihydro dinor metabolite in females (Broedbaek et al., 2011). But two other studies of the urinary levels of 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP found no difference among males and females (Morales et al., 2001; Morrow et al., 1999). In a study of 588 subjects from three countries, the urinary levels of F_2-IsoPs were modestly higher in female smokers and non-smokers versus the matched males (Basu et al., 2009). In children, basal levels of urinary IsoPs were found to be 10% higher in girls than boys (Kauffman et al., 2003).

5.11.2. Age

Age is often a non-stressor determinant in urinary levels of IsoPs and their metabolites. A study of 845 healthy women aged 40-70 years provides a typical example, where urinary F_2-IsoPs

decreased with age but urinary 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP increased with age (Dorjgochoo et al., 2011); the former could be a result of the latter.

While basal levels of urinary IsoPs are known to often increase with age of adults, they initially decrease in children up to age 5, followed by a slow increase after the age of 5 years. In the first large study of children, a sub-cohort of 342 children under the age of 7 was selected from a larger group at increased risk for developing type 1 diabetes. This study also provides one of the only data sets showing variability of urinary F_2-IsoPs in children. Levels were shown to decline from roughly 4,000 pg/mg creatinine at 9 months age, to roughly 2,100 pg/mg creatinine at 5 years, at which time they began to trend slowly upward (Kauffman et al., 2003).

Few studies have investigated correlations of urinary and of free and total plasma IsoPs as a function of both acute and chronic disease and of age. Healthy controls showed increased urinary F_2-IsoPs as age increased from 25 to 86 years, and the variations were a function of discrete age bands. The bias introduced by age may be a significant confounder with respect to interpreting the often contradictory data published regarding IsoP correlations with a variety of morbidities (Lee et al., 2009).

5.11.3. Heritability

One variable that would ordinarily determine in part the urinary IsoP levels from individuals is heritability - - the portion of phenotypic variation across a population dictated by inter-individual genetic variation. In 1,012 participants from the Framingham Heart Study, the heritability for urinary IsoP was the lowest (roughly 11%) among 10 other biomarkers of inflammation (Schnabel et al., 2009). A recent assessment of the role of genetics also found that whole-body oxidative damage (as measured by markers of nucleic acid oxidation and lipid peroxidation) was largely determined by environmental factors (Broedbaek et al., 2011). These studies rule out genetic determinants as major factors in IsoP production and excretion. Another variable affecting inter-individual variability is the enzymatic activity of lipases that liberate membrane-bound IsoPs into plasma (Kato et al., 2006).

6. Potential Confounders

6.1. Raw and cooked food as possible exogenous source of IsoPs

Because of the ubiquitous occurrence of many biomarkers, exogenous (ex vivo) sources would need to be considered as possible confounders in the interpretation of data gathered for BioSCIM. An obvious exogenous source of IsoP might be PUFA-rich foods prepared under oxidative conditions (such as fried fast foods). Given the importance of lipid peroxidation in the organoleptic and nutritional qualities of stored and cooked foods (coupled with the potential toxicity of these chemicals), it is surprising that so little research has been done on characterizing the oxidized products; some of the few studies have been unable to reach conclusions (e.g., De Leonardis and Macciola, 2006).

If present, IsoPs in ingested food could enter sewage after passing directly through the gut via the feces or after re-absorption from the gut followed by excretion. Disposal of certain leftover foods directly to sewers could serve as an additional source. The overall significance of these exogenous sources compared with excretion of endogenous quantities would have a highly variable correlation with per capita origins. This is the same problem faced by the use of creatinine as a biomarker (Daughton, 2012). Mention of IsoPs as possible products of dietary lipid oxidation is infrequent (e.g., Dobarganes and Márquez-Ruiz, 2003).

A number of studies have shown little effect on systemic IsoP levels as a result of lipid-rich diets (Richelle et al., 1999). Some evidence indicates that diet in general does not seem to be a confounder of plasma levels of IsoPs (Gopaul et al., 2000a). But few of these studies provide data showing whether IsoPs are generated directly in foods, especially in prepared foods containing PUFAs and subjected to high-temperature, oxidative conditions (such as frying). Food lipids undergo a wide array of peroxidation reactions during cooking (Márquez-Ruiz et al., 2008; Raatz et al., 2011). Some of the resulting markers of lipid oxidation (such as malondialdehyde) are known to be absorbed by the gut and confound interpretation of native levels (Mateos and Bravo, 2007).

Uncooked salmon is a fish rich in PUFAs, especially AA, EPA, and DHA. While cooking might be expected to increase IsoP formation, one study instead showed dramatic reductions. Baking (to internal temperatures of 63º C) was found to reduce 8-iso-$PGF_{2\alpha}$, and 8-iso-PG_{E2} (both present naturally in raw salmon at levels of 10-15 ng/100g) to levels below the limit of quantitation; the DHA-derived resolvins (D1 and D2) were also reduced from existing levels of 8-12 ng/100g to levels below the limit of quantitation (Raatz et al., 2011).

In a dietary study using fast food, 8-epi-$PGF_{2\alpha}$ was measured in hamburger and French fries (Gopaul et al., 2000b). The levels in French fries were only about one tenth those in the hamburger, probably because of the absence of arachidonic acid in the cooking oil whereas beef contains endogenous AA. The entire meal contained 246.7±25.4 pmol of 8-epi-$PGF_{2\alpha}$. Regardless of the portion that may have been absorbed by the gut, the systemic levels of 8-epi-$PGF_{2\alpha}$ (MW=354) remained unchanged. If the entire exogenous quantity were assumed to pass into sewage, its contribution on a mass basis would have been roughly 87 ng. While this quantity only represents one meal, it would comprise a minor input to sewage compared with the daily urinary endogenous excretion level for healthy individuals, cited previously as 500-5,000 ng/day; this range omits the unknown levels of endogenous IsoPs excreted via the feces, making the contribution from foods even less significant. Another study showed that "isoprostanes-like" chemicals can form during the autooxidation of fish oil (De Leonardis et al., 2008).

Not to be confused with the F_2-IsoPs are the analogous series of compounds produced by plants and which are probably abundant in the diet. These phytoprostanes (F_1-IsoPs) occur in higher plants (which do not synthesize arachidonic acid but rather other PUFAs) (Mueller, 2004; Thoma et al., 2004). These generally result from nonenzymatic oxidation of linolenate in plant phospholipids. F_1-Phytoprostanes occur at levels 1-2 orders of magnitude higher in plant tissues than F_2-IsoPs do in mammals.

Should exogenous sources of F_2-IsoPs prove problematic for BioSCIM, the simplest solution would be to target IsoP metabolites, such as the dinor beta-oxidation products, whose exogenous levels should be extremely low. Urinary levels of the dinor metabolites and the parent IsoP can be highly correlated. Moreover, in one study showing high correlation (r=086), the urinary basal

level of the dinor metabolite 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ was found to be roughly twice as high as the parent 8-iso-$PGF_{2\alpha}$: 506±255 vs. 223±135 pg/mg creatinine, respectively (Schwedhelm et al., 2000).

6.2. Dietary lipid influence on IsoP production

A major potential confounder might logically be the influence of PUFAs (consumed via the diet or nutritional supplements) on IsoP production. Two key PUFAs are the omega-3 fatty acids found especially in marine fish oils: EPA and DHA. Since these fatty acids are more unsaturated than arachidonic acid (AA) and therefore more prone to oxidation, it was originally believed that they might promote oxidative stress. But these omega-3 fatty acids possess significant anti-inflammatory activity from competitive interference with the cascade of inflammatory events that originate with the production of the eicosanoids (such as the prostaglandins) via the action of cyclooxygenases and lipoxygenases on arachidonic acid. EPA can replace AA in cellular membrane lipids and it can also compete for oxidants with AA. EPA has four bis-allylic carbons, versus the three of AA, where oxygen insertion can occur. The non-enzymatic oxidation of EPA has been elaborated by Brooks et al. (2008). Clearly, a diet rich in EPA has the potential to ameliorate the production of IsoPs from AA as a result of oxidative stress; excessively high doses of omega-3 fatty acids, however, can produce oxidative stress (Serini et al., 2011). And indeed, many studies show reductions in urinary F_2-IsoP of roughly 20-27% (Mori, 2004). Of course, the amelioration in IsoP production from dietary intake of EPA/DHA could also be viewed as a healthy lifestyle factor (i.e., health-favorable diet) rather than a confounder.

One of the first studies showing reductions in urinary F_2-IsoP levels after administration of omega-3 FAs began with baseline 24-h excreted totals of roughly 4,000 pmol/24h. Post-intervention levels dropped to roughly 205 to 3,000-3,400 pmol/24h (Mori et al., 1999). But the data from clinical studies using dietary interventions show mixed results. One study, involving 113 subjects receiving daily EPA/DHA supplements (from krill and fish oil) for 7 weeks, showed no changes in urinary 8-iso-$PGF_{2\alpha}$ (Ulven et al., 2011). One of the first studies to show that IsoP formation is independent of dietary intake of lipids showed that intra-individual urinary IsoP (for 4 subjects) did not vary when dietary lipid intake was changed from comprising 35% caloric intake to 5% (Richelle et al., 1999). Supplementation with EPA/DHA, however, has been found to reduce IsoP formation (Nälsén et al., 2006b). In one of the largest dietary intervention

studies of subjects with metabolic syndrome, diets varying according to the quality and quantity of unsaturated fat yielded no changes in serum IsoP levels (Petersson et al., 2010).

Dietary intervention for 20 elderly (age >65 y) but healthy patients led to a minor reduction (roughly 15%, from 6 ng/mL to 5 ng/mL) in urinary 8-epi-prostaglandin $F_{2\alpha}$ with a mono-unsaturated-enriched diet versus a diet enriched with saturated or n-3 lipids (Marin et al., 2011). Increased intake of α-linolenic acid (ALA) in the form of flaxseed oil served to increase plasma and urinary levels of F_1-phytoprostane but had no effect on plasma or urinary F_2-IsoPs (Barden et al., 2009).

For 259 healthy women, adherence to a Mediterranean diet for up to 2 menstrual cycles was found to highly correlate with reduced plasma F_2-IsoP (8-iso-$PGF_{2\alpha}$) levels (Gaskins et al., 2010).

The effect of omega-3 fatty acids on IsoPs can sometimes seem contradictory - - with levels not just decreasing or remaining unchanged (as shown with the studies cited above) but sometimes also increasing when n-3 FAs are supplemented. This contradiction stands out with one study of 48 trained cyclists supplemented with EPA and DHA as part of their daily diet. With intense, sustained exercise, plasma levels of F_2-IsoP increased 53% from baseline - - from roughly 24 to 36 pg/mL; when antioxidants were also supplemented, the levels were still elevated - - by 33%. Since EPA does not feed into the F_2-IsoP pathway, the authors proposed that the oxidized products from EPA (i.e., F_3-IsoPs) and DHA (i.e., F_4-IsoPs), as well as 4-hydroxy-2E-hexenal, were serving to oxidize AA (McAnulty et al., 2010).

In a study of 38 apparently healthy men, 18 were administered conjugated linoleic acid (CLA) during a 5-week study. Baseline urinary 8-iso-$PGF_{2\alpha}$ in both groups was roughly 1 ng/mL (0.33-2.31). At the study's conclusion, the level in the CLA group was 2.51 μg/L±0.39 versus 1.37±0.20 for the controls (Raff et al., 2008). These data support the contention that IsoP levels can appear to increase if a preferred substrate for peroxisome proliferator-activated receptors (PPAR), such as CLA is present. CLA may compete for PPAR with IsoPs, reducing the metabolism of an IsoP to its dinor metabolite (Iannone et al., 2009). Peroxisomal beta-oxidation

probably plays an important role in continually serving to reduce IsoP levels, but with concomitant increased production of the dinor metabolites (Banni et al., 2010).

6.3. Exogenous antioxidants as inhibitors of IsoP production

Ingestion of exogenous antioxidants occurs as an everyday aspect of consuming food as well as via intake of nutrition supplements. Antioxidants have the potential to reduce the production of IsoPs. A reduction in IsoP excretion from intake of antioxidants can be viewed both as a confounder and as a reflection of a behavior that can improve health.

Correlation of IsoP levels with dietary antioxidant intake is often nebulous, confusing, or equivocal (Halliwell et al., 2005). Contradictory results have long been reported from large-scale antioxidant studies on the amelioration of inflammatory diseases (Niki, 2011). In general, non-enzymic lipid peroxidation in vivo (e.g., measured as F_2-IsoPs in plasma) has been found to not be easily altered by routine changes in diet (Nälsén et al., 2006a). With regard to supplementation with individual antioxidants, most studies have focused on vitamin C (ascorbate), vitamin E (various tocopherols), lipoic acid, ubiquinone/ubiquinol, and selenium. Biomarkers of oxidative stress are also used as indirect measures of antioxidant activity in studies of antioxidant therapy (Wood et al., 2006). Some of the negative and contradictory data on IsoP production after dietary and antioxidant interventions have been discussed by Nälsén (2006). Many of the studies on antioxidants are integral parts of multifactorial studies that also examine exercise, diet, or smoking.

Results might be a strong function of the initial health status, level of exercise, and personal diets of the test subjects - - where only those with elevated baseline levels of IsoPs (or reduced endogenous levels of antioxidants) might derive any benefit from antioxidant supplementation; failure to see inverse relationships between antioxidant intake and IsoP levels is sometimes a function of insufficient doses or dosing durations (Block et al., 2008; Fisher-Wellman and Bloomer, 2009). This may simply reflect the comparative level of regulated (controlled) eustress versus unregulated (uncontrolled) overall stress. Regulatory mechanisms undoubtedly prevent IsoPs from being reduced below a certain level required for maintaining health. Antioxidants seem to have measurable effects on reducing IsoPs when other susceptibility factors exist, such as reduced anti-oxidant levels or high IsoP levels.

For many healthy individuals, further reductions in IsoP levels may not be possible and may even be unhealthy (Block et al., 2008). One example studied the effect of supplementing vitamins C and E on oxidative stress following rigorous aerobic exercise. During both pre- and post-exercise, urinary F_2-IsoPs in treated and control subjects maintained roughly in the range of 6 ng/mL (Bailey et al., 2010). A study that examined IsoP plasma levels in smokers and the effect of antioxidant therapy was perhaps the first to note that BMI plays a central role in whether IsoP levels can be therapeutically reduced - - probably because the baseline IsoP levels in those with higher BMI are much higher at the outset (Dietrich et al., 2002). Beta-carotene was not found to lower total urinary IsoPs or 8-Iso-$PGF_{2\alpha}$ in smokers or non-smokers (Mayne et al., 2004).

Some representative data include the following. Some miscellaneous data showing the kinetics of IsoP plasma reductions during antioxidant treatments are provided by Coolen et al. (2005).

Urinary 8-iso-$PGF_{2\alpha}$ was reduced by over 10% in the highest quartile of serum-selenium concentrations in 615 Swedish men over a 27-year period beginning at age 50 (Helmersson et al., 2005a). A double-blind, placebo-controlled study of the effects of vitamin E on 48 healthy but moderate smokers showed that urinary 8-iso-PGF_2 was unchanged in all treatments including doses of 1,200 mg/day (Patrignani et al., 2000). A study of 184 non-smokers followed urinary 8-isoprostaglandin F_2 during a multi-month administration of vitamin C, vitamin E, and both combined. While reductions were noted, they were not strong and no synergistic effect was noted for the two antioxidants combined (Huang et al., 2002). Administration to smokers of vitamin C (2 g/day), but not vitamin E alone, reduced urinary 8-epi-$PGF_{2\alpha}$ by 29% (Reilly et al., 1996).

High-dose supplementation of vitamin C (8 g/day), vitamin E (3,200 IU/day), and beta-carotene (240 mg/day) for 16 weeks reduced urinary 15-F -IsoPs by 45% (Morales et al., 2001). A study that examined the effect on plasma IsoP by high-level, long-term dosing of vitamin E (RRR-alpha-tocopherol) was the first to demonstrate a significant dose-dependent reduction. But the minimum dose required was 1,600-3,200 IU/day for 16 weeks. At a dose of 3,200 IU/day, reduction in 15-F_2-IsoP was 49%. These doses of vitamin E (in a natural form that is not

commonly used in clinical studies) far exceed those routinely taken or that had been used in prior clinical studies (Roberts II et al., 2007).

A double-blind study of 23 overweight and obese adults (compared with 10 healthy controls) assessed a 3-week daily regime of 5- or 20-mg doses of astaxanthin on plasma 15-F_{2t}-IsoP. While baseline levels of F_2-IsoP in test subjects were greatly elevated compared with controls (4.6-5.3 ng/mL vs. 2.5), these elevated levels were dramatically reduced (1.6-1.9 ng/mL) after 3-weeks of astaxanthin (Choi et al., 2011).

One of the first reports of an inverse relationship between plasma levels of xanthophyll carotenoids (i.e., lutein and beta-cryptoxanthin) came from a study of 37 women in a 2-week dietary intervention focused on vegetable and fruit intake. An inverse association was found for carotenoid levels and urinary 8-epi-prostaglandin $F_{2\alpha}$ despite the fact that the baseline IsoP levels were probably near baseline (Haegele et al., 2000).

In a study of type 2 diabetic patients undergoing treatment with statins, 23 subjects were also treated with coenzyme Q_{10} (CoQ_{10}) supplements. Plasma levels of F_2-IsoPs (roughly 1.3 nmol/L) did not change after treatment or differ from controls (Hamilton et al., 2009). Urinary 15-F_{2t}-IsoP levels increased less when CoQ_{10} was supplemented before and during a strenuous 50-km run (levels increased from roughly 1 ng/mL to 3.88 ng/mL compared with 5.83 ng/mL for a placebo group) (Díaz-Castro et al., 2011). For 11 subjects, supplementation with 80 mg/day of lycopene for 4 weeks did not alter urinary 8-iso-PGF levels (Ferretti and Flanagan, 1997).

A series of studies by Thompson et al. offer some of the best perspective regarding the often contradictory findings regarding IsoP excretion levels and the intake of diets rich in foods purported to reduce oxidative damage. Whether IsoP urinary output was reduced as a result of dietary intervention with antioxidant-rich foods was first proposed (in 2005) to be conditional on the baseline level of oxidative stress (Thompson et al., 2005b). A 2-week study of 106 women determined the effect of diets high in vegetables and fruits known to have high botanical diversity in terms of purported ability to minimize oxidative damage. The reduction in IsoP was most pronounced in the upper quartiles of IsoP urinary baseline levels and almost nil in the

lowest quartile. The pre-intervention mean levels ranged from 0.15-0.60 μmol/mol creatinine, while the post-intervention mean levels ranged from 0.13-0.35 μmol/mol creatinine (Thompson et al., 2006). In a prior study of 64 women, those with baseline urinary IsoP levels above the median experienced reductions of 40% after a 14-day intervention with a diet high in vegetables and fruit, but those with baseline levels below the median had no significant reductions (Thompson et al., 2005a).

A commercially available formula of highly concentrated fruits and berries was tested on 44 non-obese, non-smoking, nondiabetic, but hypercholesteremic males. Urinary 8-epi-$PGF_{2\alpha}$ decreased from 450±170 (420±165 for placebo) to 330±159 pg/mg creatinine after 4 weeks of dietary intervention (Abidov et al., 2005). For 103 healthy adults, a 3-month diet rich in fruit, vegetables, and low-fat dairy reduced urinary 8-iso-PGF_2 by roughly 10% (Miller et al., 2005).

Dietary intervention augmented with Brassica vegetables significantly reduced urinary F_2-IsoP, by 22%; reductions were greater in men than in women (25% versus 15%) (Fowke et al., 2006). Consumption of a freshly prepared vegetable soup twice a day for 2 weeks led to continual declines (reaching 27%) in plasma 8-epi-$PGF_{2\alpha}$ for six healthy men and six healthy women (Sánchez-Moreno et al., 2004). For 60 overweight men, a 6-week diet rich in antioxidants did not alter urinary IsoP (Rytter et al., 2010). For 39 children, 3-week intervention with antioxidant supplements containing dried fruits and vegetables fortified with antioxidants did not alter urinary IsoP (Stewart et al., 2002).

Although teas (green and black) are known to increase the antioxidant capacity of the blood, neither the consumption of tea nor standardized extracts has been found to influence the levels of IsoPs (Donovan et al., 2005; Rietveld and Wiseman, 2003).

Clearly, a number of interacting variables, coupled with pre-existing baseline levels of IsoPs and antioxidants, govern the range and magnitude of responses observed in these clinical studies.

Antioxidants can also act as prooxidants. One such study assessed the interaction of antioxidants with intense exercise. Alpha-tocopherol was supplemented in 38 triathletes for 2 months prior to

the Iron Man Triathlon. Plasma levels of F_2-IsoPs immediately post-race increased 181% (from roughly 28 to 75 pg/mL) in the vitamin E supplemented group versus a 97% increase in the untreated group (McAnulty et al., 2005).

6.4. Other dietary influences on IsoPs

Dietary factors other than intake of exogenous PUFAs or antioxidants can also influence IsoP production. One example is caloric restriction (coupled with increased intake of legumes), which was found to reduce the urinary 8-iso-$PGF_{2\alpha}$ in obese subjects (average BMI=35) (Crujeiras et al., 2007). But a single day of fasting was shown to significantly increase IsoP excretion (Richelle et al., 1999).

Short-term, induced hyperglycemia in 13 healthy adults led to increased urinary IsoP. Increasing plasma glucose from a baseline of 95 mg/dL to 200-250 mg/dL during a 2-h infusion led to roughly a 3-fold increase in urinary F_2-IsoPs - - from about 5 to 14 ng/mg creatinine; this was followed by a return to baseline within 24 h (McGowan et al., 2006). Plasma 15-F_{2t}-IsoP increased by 33% over baseline levels in type 2 diabetics after fructose loading (410 pg/mL versus 310 pg/mL) and by over 40% versus non-diabetics (410 pg/mL versus 290 pg/mL) (Faure et al., 2008a).

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