Introduction

According to the Centers for Disease Control and Prevention, more than 10% of adults in the United States have some form of kidney disease. Chronic kidney disease (CKD), characterized by a progressive loss in renal function over a period of months or years, is the eighth leading cause of death in the United States. Progression of CKD to end-stage renal disease (ESRD) occurs when the kidneys are no longer able to remove enough wastes and excess fluids from the body. Kidney transplantation and dialysis are the only available therapies for ESRD management. Early detection of renal disease using biomarkers may help to prevent and/or predict progression to CKD and ESRD resulting in better patient management and would be more cost-effective than highly invasive renal transplantation or dialysis procedures.

The initial diagnosis of renal disease is usually based on detection of proteinuria or an elevation of serum creatinine, freely filtered by the glomerulus, and most commonly used to determine an estimated glomerular filtration rate (eGFR), an indicator of renal function. However, use of creatinine as a diagnostic marker has limitations. Serum creatinine varies by age, race, sex, muscle mass, metabolism, nutritional status, co-morbid conditions, hydration status, and medication use and, consequently, significant renal disease can exist with minimal or no change in creatinine. Therefore, markers of early injury, especially those that correlate with early fibrosis and progression, are needed and would prove beneficial in both diagnosis and patient management settings. An ideal renal disease biomarker should be accurate, reliable, and easy to measure with a standard non-invasive, reproducible, and sensitive assay. Three biomarkers, folate receptor alpha (FRA), mesothelin (MSLN), and megakaryocyte potentiating factor (MPF), described in detail below, were selected for evaluation as potential markers of renal disease. Each of these biomarkers is currently being evaluated as potential diagnostics in oncology and each is known to be filtered by the kidneys. As such,
we reasoned that they may serve as viable candidate markers of renal function. Robust and reproducible assays are available for all three biomarkers.

FRA is a 38–40 Da glycosylphosphatidylinositol (GPI)-anchored protein that binds plasma folate (5-methyltetrahydrofolate) with high affinity (K_D ~1nM) and transports it into the cell via endocytosis. In normal human tissues, FRA expression is restricted to polarized epithelial cells in a number of tissues,\textsuperscript{10–13} including high expression in the proximal tubules of the kidney where it has been shown to play a role in the tubular reabsorption of folate into the circulation.\textsuperscript{14} Therefore, impairment of kidney function may prevent reabsorption of folate into the circulation and could cause folate deficiency,\textsuperscript{15} which might then result in upregulation of FRA synthesis.\textsuperscript{16–20} FRA has also been shown to be expressed on a number of epithelial tumors including ovarian, endometrial, lung adenocarcinoma, renal clear cell cancer, and triple negative breast cancer.\textsuperscript{10–13,21–26}

MSLN is a 70 kDa protein, which upon proteolytic cleavage results in a 32 kDa secreted product termed MPF and MSLN, a 40 kDa GPI-anchored glycoprotein that is also shed into the circulation.\textsuperscript{29,30} Serum MSLN is currently used for mesothelioma diagnosis, prognosis, and monitoring.\textsuperscript{31–33} MSLN is hypothesized to be involved in cell adhesion through interactions with MUC16 and intracellular signaling and is highly expressed in ovarian cancer,\textsuperscript{34–37} mesothelioma,\textsuperscript{34,37–39} pancreatic cancer,\textsuperscript{37} and a subset of lung adenocarcinoma.\textsuperscript{37} MSLN also demonstrates restricted expression in normal tissues and is primarily expressed on mesothelial cells of the peritoneum, pericardium, and pleura.\textsuperscript{34,37,40} Little is known about the biological function of MPF. However, given that it is a cleavage product from the MSLN precursor protein, its expression should mimic that of MSLN. Owing to its value as a diagnostic for mesothelioma, some studies on MSLN relative to kidney function have been reported. Studies have shown that serum MSLN levels were increased in individuals with renal impairment and that these levels were dependent on the stage of CKD.\textsuperscript{17,31,41,42} Further, Hollevoet et al (2010) showed that an increase in MSLN correlated with an increase in creatinine levels.\textsuperscript{31}

While some literature on the variation of serum MSLN levels relative to kidney function exists, no such literature exists to our knowledge on serum MPF or FRA, and no comparison relative to the urinary levels of these markers is available. A urine-based assay would of course be ideal as a diagnostic or monitoring tool as it requires totally non-invasive procedures for sample acquisition. As such, we undertook a preliminary investigation of the potential clinical utility of measurements of FRA, MSLN, and MPF, in serum and urine, as an aid in the diagnosis or assessment of renal disease. The results indicate that these markers may indeed have potential value in predicting progression in subjects with renal disease and are worthy of further study. Further, the demonstration of significant changes in the serum concentrations of these biomarkers relative to renal function may have implications to their use in oncology diagnostics, in particular, monitoring of disease, as some chemotherapeutics are known nephrotoxics.

**Methods**

**Subjects samples.** This preliminary study included matched serum and urine samples from 200 subjects with varying stages of renal disease and 100 age-matched healthy subjects (Table 1). Samples were obtained from various commercial vendors with Institutional Review Board approvals and patient consent and were collected between 2009 and 2011 by standard techniques and processed/frozen within 30 minutes of collection. Urine samples represented spot collections collected at the time of blood draw and were centrifuged prior to freezing. All samples were stored at ~80 °C, and thawed and aliquoted prior to analysis. Patient demographics, including sex, race, age, and stage (Table 1) were obtained from the suppliers.

**Electrochemiluminescence (ECL) assays.** The ECL assays for FRA, MSLN, and MPF used in the present analyses have been described previously.\textsuperscript{43,44} Samples (serum, urine) from healthy or diseased subjects and standards were added to wells of 96-well plates previously coated with marker specific capture monoclonal antibody (MAb) and incubated at room temperature. A capture monoclonal antibody and an appropriate conjugate were added to each well. After gentle mixing, plates were incubated for 1 hour at room temperature. This was followed by washing and the addition of a second monoclonal antibody for detection of captured MAb labeled with a reporter moiety. Antigen-antibody complexes were detected using a sandwich ECL immunoassay with the standard procedure for the capture monoclonal antibody (MAb) and incubated at room temperature. The ECL signal was captured with a microplate reader and the data was analyzed by software using the standard curve obtained for each test.

**Table 1. Demographic and clinical characteristics of healthy subjects and renal disease subjects.**

| VARIABLE                        | HEALTHY SUBJECTS N (%) | RENAL DISEASE SUBJECTS N (%) |
|---------------------------------|------------------------|-------------------------------|
| Total sample size               | 100                    | 200                           |
| Age (Mean, SD)                  | 60.2, 6.9              | 67.9, 13.7                    |
| Gender                          |                        |                               |
| Male                            | 40 (40)                | 82 (41)                       |
| Female                          | 60 (60)                | 118 (59)                      |
| Race                            |                        |                               |
| White/Caucasian                 | 51 (51)                | 123 (61.5)                    |
| Black/African American          | 38 (38)                | 14 (7)                        |
| Native American/Alaskan         | 0                      | 1 (0.5)                       |
| Asian                           | 4 (4)                  | 2 (1)                         |
| Hispanic                        | 0 (0)                  | 7 (3.5)                       |
| Other                           | 0 (0)                  | 53 (26.5)                     |
| Unspecified                     | 7 (7)                  | 0 (0)                         |
| renal disease stage\textsuperscript{\textsuperscript{1,2}} |                        |                               |
| Stage III                       | 124 (62)               |                               |
| Stage IV                        | 22 (11)                |                               |
| Stage V                         | 4 (2)                  |                               |
| Unspecified                     | 50 (25)                |                               |

Notes: Numbers and percentages exclude healthy subjects. \textsuperscript{1}GFR was used in the determination of renal disease stage.
time for two hours. The ruthenium labeled detection MAbS were diluted in assay buffer, added to washed plates, and incubated for an additional two hours at room temperature. Plates were washed, read buffer added, and signals measured using an MSD DISCOVERY WORKBENCH® (Mesoscale Discovery, Gaithersburg, MD). Optimal sample dilutions were: FRA (80-fold dilution of urine and a 20-fold dilution of serum), MSLN (60-fold dilution of urine and an 80-fold dilution of serum), and MPF (4-fold dilution of urine and a 20-fold dilution of serum).

**Creatinine analysis.** Creatinine concentrations in serum were determined using the QuantiChrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA).

**Statistical analyses.** Pearson’s correlation coefficient was performed to determine the correlation between the various biomarkers. Pairwise comparisons of biomarker levels between healthy subjects and stages of renal disease were performed using ANOVA followed by Tukey’s post-hoc analysis. Receiver operating characteristic (ROC) analysis was employed to determine the performance of each marker by presence and stage of disease. ROC area under the curve (AUC) calculations were based on 95% confidence intervals. All comparisons were two-sided and a P-value ≤0.05 was considered statistically significant except where otherwise stated. Statistical analyses were performed in GraphPad Prism version 6.00 (GraphPad Software, Inc., La Jolla, CA).

**Results**

The ECL assays for FRA, MSLN, and MPF had intraday variability between 2–16% and excellent sensitivity with lower limits of detection (LLOD) of 1.22, 0.29, and 3.35 pg/mL, for FRA, MSLN, and MPF, respectively.43

Matched serum/urine sample pairs from healthy subjects and subjects with renal disease were measured for FRA, MSLN, and MPF using the described ECL assays and for creatinine using a commercial assay. The patient cohort investigated in the present study was limited and skewed toward stage III disease making some comparisons difficult.

Importantly, all three protein markers were readily detected in both serum and urine matrices. A Pearson’s correlation matrix for the serum and urinary levels of these protein biomarkers and serum creatinine in both healthy subjects and subjects with renal disease is presented in Table 2. No significant correlation was observed between serum and urine levels for any of the three protein biomarkers, with correlation coefficients of r = −0.02–0.22 (Table 2) in healthy subjects. There was no correlation between serum and urine FRA levels in subjects with renal disease and a moderate to strong correlation between serum and urine MSLN and MPF levels in subjects with renal disease.

As can be seen, in healthy subjects and in subjects with renal disease, serum FRA was moderately correlated with MSLN and MPF and, as expected, MSLN was strongly correlated with MPF since those two proteins derive from the same gene product through proteolytic processing. Creatinine only weakly correlated with FRA, MSLN, or MPF. Further, no significant correlation was noted for any of the three protein biomarkers relative to age or gender (data not shown).

Serum levels of FRA, MSLN, MPF, and creatinine are shown in Table 3 and demonstrate a highly significant

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**Table 2.** Pearson’s correlation coefficients1 between biomarkers in urine (u) and serum (s) from healthy subjects or renal disease subjects.

| BIOMARKER | HEALTHY SUBJECTS | RENAL DISEASE SUBJECTS |
|-----------|-----------------|-------------------------|
| **sFRA**  | sMSLN | sMPF  | sCREAT | uFRA  | uMSLN | uMPF  |
| sFRA  | 1     | 0.68*** | 0.74**** | 0.56*** | 0.02  | 0.02  | 0.32** |
| sMSLN | 0.68*** | 1     | 0.75**** | 0.47*** | 0.04  | 0.22* | 0.38** |
| sMPF  | 0.74*** | 0.75**** | 1     | 0.31**  | −0.08 | 0.03  | 0.12  |
| sCreat | 0.56*** | 0.47**** | 0.31** | 1     | 0.01  | 0.22* | 0.76**** |
| uFRA  | 0.02  | 0.04  | −0.08 | −0.01 | 1     | 0.45** | −0.02 |
| uMSLN | 0.02  | 0.22* | 0.03  | 0.22*  | 0.45** | 1     | 0.22* |
| uMPF  | 0.32** | 0.38** | 0.12  | 0.76**** | −0.02 | 0.22* | 1     |

**Notes:** Correlations were classified as follows: ****very strong, ***strong, **moderate, *weak.
Biomarker insights 2014:9 – Table 0.75, 2343.22 mean, SD Fig. 26138.54 – 0.86, 0.73 P 18999.45, 14238.76 668.07 mean, SD Fig. RenAL DiSeASe SubjeCtS 3–9 Fig. 671.62, 30462.65 140.47, 2173.20 7198.91, 132260.13 1130.17, 18608.72 mean, SD Fig. 4716.55, 2746.52 Fig., 31752.16, 19897.75 2.11, 2.26 0, 7.09 Fig. 4103.03 366.35, 251.39, (n 860.49, 581.81 – StAtiStiCS 243.15, 4296.04 2957.77, 3240.11 progression. The ROC analyses presented for these four markers combination, of markers may be useful in quantifying disease pro with increasing stage of disease suggesting that one, or a com (Serena et al., 2007). FRA and MPF and MSLN were also shown to increase in both renal disease subjects and healthy subjects with 4). These data suggest that additional studies aimed at combining a number of markers, including those described herein, may yield higher diagnostic accuracy and monitoring potential for renal disease.

**Discussion**

Creatinine remains the gold standard in the diagnosis and monitoring of impaired kidney function due to acute kidney injury (AKI), CKD, or subsequent to renal transplantation. Nevertheless, creatinine as a diagnostic marker has limitations, with levels being affected by many factors. It also support the potential of a combination of markers for evaluation of disease progression as each marker varies in its ability to distinguish between stages of disease. Clearly more work is warranted to further establish the clinical value of a multi-marker panel. Furthermore, FRA (Fig. 3B) and creatinine (Fig. 3H) were able to accurately distinguish stages 3, 4, and 5 of renal disease. MSLN (Fig. 3D) and MPF (Fig. 3F) poorly distinguished stage 3 of renal disease, but were able to distinguish stage 4 and stage 5 of renal disease.

These data suggest that additional studies aimed at combining a number of markers, including those described herein, may yield higher diagnostic accuracy and monitoring potential for renal disease.

**Table 3.** Serum biomarker levels for healthy subjects and renal disease subjects.

| BIOMARKER | STATISTICS | HEALTHY SUBJECTS (N = 100) | RENAL DISEASE SUBJECTS (N = 200) | P-VALUE |
|-----------|------------|---------------------------|---------------------------------|---------|
| FRA (pg/mL) | Mean, SD | 366.35, 251.39 | 860.49, 581.81 | <0.0001 |
| Median | 313.86 | 668.07 |
| Min, Max | 140.47, 2173.20 | 243.15, 4296.04 |
| MSLN (pg/mL) | Mean, SD | 18999.45, 14238.76 | 31752.16, 19897.75 | <0.0001 |
| Median | 14946.09 | 26138.54 |
| Min, Max | 6065.98, 83304.74 | 7198.91, 132260.13 |
| MPF (pg/mL) | Mean, SD | 2957.77, 3240.11 | 4716.55, 2746.52 | <0.0001 |
| Median | 2343.22 | 4103.03 |
| Min, Max | 671.62, 30462.65 | 1130.17, 18608.72 |
| Creatinine (mg/dL) | Mean, SD | 0.86, 0.73 | 2.11, 2.26 | <0.0001 |
| Median | 0.75 | 1.36 |
| Min, Max | 0.709 | 16.82 |

(P < 0.0001) discrimination between healthy subjects and renal disease subjects, evaluated as a single cohort. These data are shown graphically in Figure 1: FRA (Fig. 1A), MSLN (Fig. 1B), MPF (Fig. 1C), and creatinine (Fig. 1D). Further, urinary levels of FRA (Fig. 2A), MSLN (Fig. 2B), and MPF (Fig. 2C) were also shown to discriminate between subjects with renal disease and healthy subjects with P = 0.0004 for FRA and MPF and P < 0.0001 for MSLN (Table 4). These data demonstrate that while urinary measurements of these biomarkers are discriminatory, they appear somewhat less sensitive than serum determinations that may reflect inherent difficulties in analysis of urine samples and/or the fact that these were spot urine collections. This is reflected in the ROC analysis of urine data (Fig. 2D), which shows AUC values of: FRA = 0.68 (P < 0.0001); MSLN = 0.72 (P < 0.0001); and MPF = 0.60 (P = 0.003). However, these findings suggest further work is warranted to better understand the urinary excretion of these biomarkers, especially with respect to standardization of urine collections. Although little is known of the biological function of this growth factor, it is interesting to speculate that urinary MPF is not only a reflection of glomerular filtration per se, but also of an altered inflammatory environment.

ROC analysis (Fig. 1E) of serum levels of these markers resulted in AUCs of 0.89 (P < 0.0001) for FRA, 0.76 (P < 0.0001) for MSLN, 0.79 (P < 0.0001) for MPF, and 0.88 (P < 0.0001) for creatinine. These data suggest that serum determinations of FRA perform similarly to the standard serum creatinine measurements and may therefore be useful in the diagnosis of renal disease. In addition, serum determinations of FRA (Fig. 3A), as well as MSLN (Fig. 3C), MPF (Fig. 3E), and creatinine (Fig. 3G) were shown to increase with increasing stage of disease suggesting that one, or a combination, of markers may be useful in quantifying disease progression. The ROC analyses presented for these four markers (Fig. 3B (FRA), 3D (MSLN), 3F (MPF), and 3H (creatinine)) also suggest the potential of a combination of markers for evaluation of disease progression as each marker varies in its ability to distinguish between stages of disease. Clearly more work is warranted to further establish the clinical value of a multi-marker panel. Furthermore, FRA (Fig. 3B) and creatinine (Fig. 3H) were able to accurately distinguish stages 3, 4, and 5 of renal disease. MSLN (Fig. 3D) and MPF (Fig. 3F) poorly distinguished stage 3 of renal disease, but were able to distinguish stage 4 and stage 5 of renal disease.

These data suggest that additional studies aimed at combining a number of markers, including those described herein, may yield higher diagnostic accuracy and monitoring potential for renal disease.
The three biomarkers showed moderate to strong correlations with each other in serum, with Pearson's correlation coefficients ranging from 0.68 to 0.75. MSLN correlated well (Pearson's coefficient = 0.75) with MPF, but this is not surprising since these two proteins derive from the same gene product. There is no known biological connection between FRA and MSLN except for the fact that they are both GPI-anchored proteins. As such, it can be speculated that the relatively high correlation between the serum levels of these two proteins reflects a more generalized biologic process related to clearance of these two molecules. While MSLN has been shown to be a binding partner for Muc16 with unknown consequences on its circulatory half-life, FRA has been shown to bind to megalin in both the kidney and liver.
Table 4. Urinary biomarker levels for healthy subjects and renal disease subjects.

| BIOMARKER | STATISTICS | HEALTHY SUBJECTS (N = 100) | RENAL DISEASE SUBJECTS (N = 200) | P-VALUE |
|-----------|------------|-----------------------------|----------------------------------|---------|
| FRA (pg/mL) | Mean, SD   | 12652.86, 18824.77          | 21658.97, 23872.92               | 0.0004  |
|           | Median     | 7737.11                     | 14558.71                         |         |
|           | Min, Max   | 122.71, 127187.14           | 186.33, 158325.80                |         |
| MSLN (pg/mL) | Mean, SD   | 2479.55, 4555.67           | 7136.96, 9418.64                 | <0.0001 |
|           | Median     | 844.44                      | 3993.69                          |         |
|           | Min, Max   | 0, 32318.28                 | 2.26, 63141.51                   |         |
| MPF (pg/mL)  | Mean, SD   | 78.22, 267.97              | 735.89, 2542.52                  | 0.0004  |
|           | Median     | 15.28                       | 32.37                            |         |
|           | Min, Max   | 0, 2389.48                  | 0, 17164.88                      |         |
Figure 3. Scatter plots of the serum levels of: (A) FRA, (C) MSLN, (E) MPF, and (G) creatinine in healthy subjects and renal disease subjects by stage of disease. Data is plotted on a log scale. The line and error bars depict mean and standard deviation, respectively. P-values reflect differences between each stage of renal disease and healthy subjects. ROC curves showing the performance of: (B) FRA, (D) MSLN, (F) MPF, and (H) creatinine in discriminating healthy subjects from renal disease subjects by stage (III, red line; IV, blue line; V, green line). AUC values for each ROC are depicted; P-values reflect differences between the AUC of healthy subjects and renal disease subjects with various stages of disease.
in fact filtered by the kidneys and may, therefore, lend themselves to non-invasive sampling techniques for the diagnosis and/or monitoring of renal dysfunction. Of particular note is urinary MPF, which is largely undetectable in healthy subjects but can reach quite high levels in subjects with renal disease.

When measured in serum, each of the biomarkers investigated showed significant associations with increasing stage of disease, although the level of significance varied. However, further work is required to validate these proteins as potential biomarkers of value for renal disease progression. Unfortunately, the present study was limited by the distribution of stages within the patient cohort and as such should be considered preliminary in nature. Since FRA is expressed at high levels in the proximal tubules, it is interesting to speculate that the increased levels of FRA observed in the serum of subjects with renal disease may be a direct reflection of tubule damage rather than a consequence of or in addition to decreased glomerular filtration per se and further studies are warranted to elucidate the mechanism of increased serum FRA relative to renal disease.

The three protein biomarkers described herein, namely FRA, MSLN, and MPF, have been evaluated as biomarkers in ovarian cancer, pancreatic cancer, and a subset of lung adenocarcinoma. In the present work, serum levels of these three markers are described to be impacted by impaired renal function, thus suggesting their use as diagnostic biomarkers in cancer need to be evaluated cautiously. Further studies are required to elucidate the relationship between serum and urine concentrations of these markers relative to eGFR and disease stage, including the possibility that renal disease of different pathologies, eg glomerular versus interstitial, might result in differential levels of one or more of these markers, and importantly, the impact of renal function on the clinical value of these markers in cancer.

Finally, renal disease is a complex family of diseases involving multiple pathophysiological processes. As a result, a number of biomarkers spanning the known causes of/processes related to kidney disease have been investigated to various degrees. These include markers related to impairments in renal function (Cys-C), oxidative stress, [γ-Glutamyl transpeptidase (GGT)], inflammation or fibrosis, metabolic factors, [Apolipoprotein A-IV, fibroblast growth factor-23], and damage to the kidney structure. [Liver-type fatty acid-binding protein (L-FABP)], kidney injury molecule (KIM)-1, neutrophil gelatinase-associated lipocalin (NGAL), and/or monitoring of kidney function. Panels of biomarkers may be the only solution to this problem and the markers described herein should be considered potential candidates for inclusion in such a diagnostic panel.

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Author Contributions
ES, DO and JV contributed to the writing of the MS. ES and DO conceived and designed the experiments. DO analyzed the data. DO wrote the first draft of the manuscript. ES and DO contributed to the writing of the manuscript. ES and DO agree with manuscript results and conclusions. ES and DO jointly developed the structure and arguments for the paper. ES and DO made critical revisions and approved final version. All authors reviewed and approved of the final manuscript.

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