

## Article

# Urine LOX-1 and volatilome as promising tools towards early detection of renal cancer

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**Simple Summary:** Renal cell carcinoma (RCC) is often late diagnosed in advanced stage, worsening the prognosis of the patients; thus an early marker is desirable. Here, we developed an innovative combined approach useful to identify for the first time the presence of LOX-1 protein within urine of clear cell RCC patients. The protein, related to metabolic disorder-associated carcinogenesis, results to be quantitatively correlated to tumor grade and stage. The analysis of volatile compounds released by urines shows the diagnostic potentialities of volatilome and indicates that at least one volatile compound is correlated with both LOX-1 and cancer.

Thus, in this work we propose the potential use of a non invasive approach enabling an early and routinely ccRCC diagnosis and focused to a better management of the patients.

**Abstract:** Renal cell carcinoma (RCC) represents around 3% of all cancers, within which clear cell RCC (ccRCC) are the most common type (70–75%).

The RCC disease regularly progresses asymptotically and upon presentation is recurrently metastatic, so an early method of detection is necessary. The identification of one or more specific biomarkers measurable in biofluids (i.e. urine) by combined approaches could surely be appropriate for this kind of cancer, especially due to easy obtainability by non invasive method.

OLR1 is a metabolic gene that encodes for the Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), implicated in inflammation, atherosclerosis, ROS and metabolic disorder-associated carcinogenesis. Specifically, LOX-1 is clearly involved in tumor insurgence and progression of different human cancers.

This work reports for the first time the presence of LOX-1 protein in ccRCC urine and its peculiar distribution in tumoral tissues. In parallel, urine samples headspace has been analyzed for the presence of the volatile compounds (VOCs) by SPME-GC/MS and gas sensor array. In particular, it was found by GC/MS analysis that 2-Cyclohexen-1-one,3-methyl-6-(1-methylethyl)- correlates with LOX-1 concentration in urine.

Thus, the combined approach of VOCs analysis and protein quantification could lead to promising results in terms of diagnostic and prognostic potential for ccRCC tumor.

**Keywords:** clear cell renal cell carcinoma (ccRCC), LOX-1 protein, volatile compounds (VOCs), prognostic biomarker, urine, gas chromatography mass spectrometer (GC/MS).

## 1. Introduction

Renal cell carcinoma (RCC) is the seventh most common malignancy among men and the ninth among women in Europe [1,2] and its incidence has increased about 2% per year in the last two decades. In 2018, about 100,000 new RCC cases and 40,000 RCC-related deaths were registered within the European Union [2]. There is a 1.5:1 predominance in men over women, with a peak incidence occurring between 60 and 70 years of age [3]. Risk factors for the development of RCC include smoking, obesity, poorly controlled hypertension, diet and alcohol, together with exposure to environmental agents (i.e., cadmium, benzene, trichloroethylene, and asbestos) [4]. Several hereditary RCC types also exist, with von Hippel-Lindau (VHL) disease being the most common [4,5].

Most cases of RCC are discovered incidentally on imaging needed due to symptoms or conditions unrelated to cancer, with about one-third of cases diagnosed when RCC has spread with distant metastases, and a further 20-50% will progress to metastatic disease despite surgical resection [6]. In recent years, survival of patients diagnosed with RCC has improved mainly due to an increase in incidentally detected RCCs at earlier stages, to better surgical techniques and to the availability of several targeted anticancer agents (mainly kinase inhibitors and immune checkpoint inhibitors) [7,8]. However, despite several biomarkers, both in blood and urine, have been suggested as leading to early diagnosis of RCC, none of them is reliable enough to be used in clinical practice [8]. Therefore, the evaluation of new potential molecules allowing early detection of RCC remains a challenging research area. To this aim, a better knowledge of the molecular components playing a key role in the carcinogenesis of RCC would help in selecting the most suitable biomarker(s) for its early diagnosis.

RCC is a complex disease presenting with different histologies, including clear cell carcinoma (ccRCC; 70-80%), papillary (type I and II, 10%), chromophobe carcinoma (5%), and other rare ones (<5%). ccRCC is characterized by genetic mutations in factors governing the hypoxia signaling pathway (HIF), resulting in metabolic dysregulation, heightened angiogenesis, intratumoral heterogeneity and deleterious tumor microenvironmental (TME) crosstalk. It occurs in both a sporadic (nonfamilial) and a familial form, such as Von Hippel Lindau Syndrome (VHL), in which the HIF transcription factors upregulate the expression of several growth factors (VEGF, PDGF, and TGF $\alpha$ ), inducing angiogenesis, proliferation and migration, as well as the expression of numerous genes regulating glucose metabolism and oxygen transport and metabolism [9]. In fact, widespread metabolic reprogramming in glucose, lipid, and amino acid metabolism broadly contributes to the clear cell phenotype [10-14].

Both oxidative and inflammatory conditions constitute major risk factors for tumour development. Reactive oxygen species (ROS) induce oxidative stress, leading to several pathological disorders due to the damage of proteins, DNA and lipids [15,16]. Obesity enhances oxidative stress by increasing ROS concentration [17] and in turn the disrupted redox balance impacts several signalling pathways associated with cell proliferation, apoptosis, invasiveness, drug-resistance and energy metabolism [18-20].

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is one of the genes involved in pathological processes such as atherogenesis, metabolic diseases, hypertension and tumorigenesis [21-22]. LOX-1 is a receptor protein, responsible for the recognition, binding and internalization of ox-LDL. It represents the most relevant lipid metabolic genes consistently overexpressed in diverse cancer cell lineages, contributing to cellular transformation by activating NF- $\kappa$ B and the inflammatory pathway [23] and in the same time to the maintenance of the transformed state. LOX-1 is primarily expressed in vascular

cells and vasculature-rich organs, but its expression is upregulated by ox-LDL, inflammatory cytokines, oxidative stress, vasoconstrictive peptides and shear stress [24]. When overexpressed, LOX-1 is able to activate in turn HIF-1 $\alpha$  and increase the expression of VEGF, MMP-2, and MMP-9, inducing the neoangiogenic and the epithelial-mesenchymal transition process [25-28]. Accordingly, both the risk factors, oxLDL and its receptor LOX-1, have also been implicated in many aspects of cancer in several tissues, e.g. glioblastoma, osteosarcoma prostate, colon, breast, lung and pancreatic [29-31].

Anyway, the role of LOX-1 is not as a direct responsible for cancer, but it supports its function in tumor progression through the combination of specific molecular pathways. Thus, due to the well known mechanistic overlap existing in the pathobiology of atherogenesis and tumorigenesis [21,22,32], LOX-1 could represent a predisposing factor for several types of cancers.

RCC is increasingly recognised as a 'disease of cell metabolism', in which oncometabolites, once aberrantly accumulated, can contribute to tumorigenesis and influence tumour phenotype and progression [23,33]. It is then likely to hypothesise that a metabolomics approach may provide the basis for a timely diagnosis of ccRCC. To explore this possibility we investigated the volatile metabolites and searched for reliable biomarkers in urines. Urines are straightforward choice for metabolomics studies in urinary tract diseases and furthermore they are easily collected and stored for successive analysis. The search of biomarkers in urines have been driven by the observation that primary events in tumorigenesis are represented by the activation of lipid metabolism, together with diabetes, metabolic syndrome and atherosclerosis.

The volatile fraction of the metabolome, the volatolome, is gaining a growing interest because of a supposed simplicity of samples collection, the intrinsic non-invasiveness of measurements and the wide availability of analytical methods. Studies evidenced that patterns of volatile organic compounds (VOCs) have been shown to be related to a vast range of phenomena observable in vitro, even at single cell level [34], and in vivo [35]. Several instrumental techniques are available for the analysis of volatolome. Gas chromatograph and mass spectrometers provides a thorough investigation about the volatolome composition. On the other hand, portable and easy to use instruments based on sensors arrays (so-called electronic noses) are also becoming available. Electronic noses have been demonstrated to be sufficiently sensitive and selective to identify diseases analyzing various human samples such as breath [36], urine [37] and sweat [38].

The interplay between LOX-1 and volatile compounds was previously observed in cell cultures [39] and in tumor xenografts in murine models [40]. In both cases the knockdown of LOX-1 resulted in a strong alteration of the pattern of volatile compounds. The changes are large enough to be also detected by an array of gas sensors. Moreover previous studies shown that RCC induces alteration of VOCs profile urines [41,42].

In this paper we investigated the expression of LOX-1 protein and the pattern of volatile compounds in the urines of ccRCC patients. The comparison with a control group revealed the diagnostic properties of this approach, resulting an alternative and innovative method for ccRCC diagnosis.

LOX-1 protein shows a peculiar distribution, exclusively revealed in tumor and peritumoral tissues. Moreover both localization and quantitative expression result to be correlated with the tumor grade. Consequently, this scenario has a proportional relapse on the protein quantity revealed in urine samples, in which LOX-1 is gradually excreted. Importantly, GC/MS results are in good agreement with previous studies of urine volatolome for RCC diagnosis [41,42]. The identification of RCC volatolome has also been carried out also by a gas sensor array, revealing the presence of a specific compound correlated to LOX-1 concentration. Altogether, this approach results as promising for the development of low-cost volatolome based diagnosis of RCC.

Importantly, this work reports for the first time the presence of LOX-1 protein in urine, pointing out LOX-1 as a potential and non invasive prognostic marker in ccRCC.

## 2. Materials and Methods

### 2.1 Patients

Fourty clear cell Renal Cell Carcinoma (ccRCC) were collected Regina Elena National Cancer Institute of Rome between November 2018 and August 2019, irrespectively of the clinical staging. Patients included in the study were not treated with any neo-adjuvant therapy before surgery. The mean age of the patients at the time of surgery was 62.1 (range 26-85), male represents the 43% and female the 57%, respectively. All specimens used in this study underwent histological examination according to 2019 WHO Classification to confirm the diagnosis. The mean size of tumor was 5.3 cm (range 0.7 – 13 cm). According to ISUP 2013 grading system, we found eight G1 cases, twenty-three G2 cases, nine G3 cases. A database was created, collecting for each patient demographic, surgical, histological and oncological informations, including also risk factors and clinical comorbidities such as diabetes, hypertension, dyslipidemia, BMI, smoking habits and the presence of autoimmune diseases. Moreover, normal renal tissues of healthy people (n=8) were collected from autoptic examination (performed within six hours from death), resulting negative for any neoplasia and renal diseases at the macroscopic evaluation and was used as control. For each patient distant normal peritumoral tissues (NpT) were also collected and analyzed, as well as neoplasia. Histological classification was carried out on haematoxylin and eosin-stained slides. All specimens were formalin-fixed and paraffin embedded. Finally, first morning urine specimens were collected at the time of surgery from all the patients and stored at 4°C until processing. The control group consisted of administrative employees (median age 60) working at the University of Tor Vergata, recruited on a voluntary basis after a signed informed consent, during the last annual health surveillance program. First morning mid-stream clean catch urine samples have been collected. Exclusion criteria are positive medical history of kidney diseases, hypercholesterolemia, hypertension, insulin resistance, diabete, urinary infections, haematuria. The study was approved by Policlinico Tor Vergata Ethical Committee (n. prot. 0023724/2014).

### 2.2 ccRCC cell line and immunocytochemistry (ICC)

Human ccRCC cell line 786-O (ATCC: CRL-1932) was used and grown in Dulbecco's modified Eagle Medium (DMEM, GE healthcare, Milan, IT) supplemented with 10% fetal bovine serum (FBS) (Euroclone, Milan, IT), Glutamine (Euroclone, Milan, IT), non-essential Amino Acids (Gibco, Life Technologies Corporation, Carlsbad, CA, USA), Penicillin-Streptomycin (Gibco, Life Technologies Corporation, Carlsbad, CA, USA). Cells were plated in 4 well/chamberslides at a concentration of 10000 cells/cm<sup>2</sup>, 3500 cells/cm<sup>2</sup> and 10000 cells/cm<sup>2</sup> respectively. After an overnight culture, the medium was removed and cells were fixed in formalin 10% for 5 minutes and stored at 4°C in PBS1X, to perform ICC analysis. Cells were permeabilized with 0,5 % Triton X-100 and 0,05 % Tween-20 in PBS1X. Afterwards, cells were washed two times with PBS1X and non-specific sites were blocked with serum incubation (ScyTek Laboratories, Super Block) for 5 minutes. Without washing, cells were incubated with primary antibodies, diluted in TBS1X/BSA 2%, for LOX-1 (Abcam) and VEGF-A (Santa Cruz)) detection. To assess the background staining, a negative control was carried out without addition of primary antibody (data not shown). After 1 hour and three washes in TBS1X/0,05% Triton X-100, cells were incubated for 15 minutes at room temperature with secondary antibody (ScyTek Laboratories, UltraTek Anti-Polyvalent Biotinylated Antibody). After repeating three washes as above, cells were incubated with Streptaividine solution (ScyTek Laboratories, UltraTek HRP) for 15 minutes. Finally, after the additional three washes, cells were incubated with 3-Amino-9-Ethylcarbazole (AEC) for signal detection, and counterstained with haematoxylin.

### 2.3 Immunohistochemistry (IHC)

Serial 5  $\mu$ m thick sections from formalin-fixed and paraffin-embedded specimens were deparaffinized and rehydrated through xylene and alcohol. Endogenous peroxidases were blocked in methanol solution with 3% hydrogen peroxide for 20 minutes; after which, sections were placed in water for 5 minutes. Subsequently, sections were placed for three times in washing solution TBS1X/0, 1% Tween-20 (5 minutes each time). Then, sections were incubated for 5 minutes at room temperature with serum (ScyTek Laboratories, Super Block) for blocking non-specific sites, and later with LOX-1 (Abcam, ab60178) and VEGF-A (Santa Cruz, sc-7269) primary antibodies, diluted in TBS1X/BSA 2%. After 1 hour, sections were washed with TBS1X/0, 1% Tween 20 solution as above, and then incubated for 15 minutes at room temperature with secondary antibody (ScyTek Laboratories, UltraTek Anti-Polyvalent Biotinylated Antibody). After repeating three washes, sections were incubated with Streptavidine solution (ScyTek Laboratories, UltraTek HRP) for 15 minutes. The staining was completed after a short incubation with a freshly prepared substrate-chromogen, 3-Amino-9-Ethylcarbazole (AEC) (ScyTek Laboratories). Sections were washed extensively in water and nuclei were counterstained with hematoxylin. The intensity of LOX-1 and VEGF-A staining was scored as negative/weak (-), moderate (+), strong (++)

### 2.4 Quantitative measurement of urine LOX-1 levels

Fresh first morning urine samples (50 mL) were collected in sterile containers and processed within 1h after collection. Urinary cells and debris were removed by centrifugation at 2000 g for 30 min at 4°C, obtaining the first fraction cell-free urine. Then, only 50 mL of the collected supernatant were transferred to clean tubes and centrifugated at 10.000 g for 30 min at 4°C to eliminate large microvesicles. After this centrifugation, the urine supernatant was added to a Vivaspin centrifugal concentrator (Sartorius) and then centrifugated at 3000 g for 30 min, in order to concentrate urine proteins.

Human LOX-1 levels were assessed from human urine RCC (n=30) and ctr (n=25) with Human Lectin like Oxidized Low Density Lipoprotein Receptor1 (LOX-1) ELISA kit (Mybiosource MB52703808), in accordance with the manufacture's instructions. The absorbance was measured with the spectrophotometer Multimode detector DTX 880 (Beckman Coulter, Milan, Italy).

### 2.5 Western blot of LOX-1 in urine

An equal proportion of the original urine volume was loaded in 2X Laemmli buffer with 40mM of dithiothreitol (DTT) on 10% SDS gels. Protein were transferred to a PVDF membrane (Hybond P, Amersham GE Healthcare, Chalfont St. Giles, UK). Anti LOX-1 (Abcam, ab60178) mouse monoclonal was used as primary antibody. Peroxidase conjugated secondary antibodies were used. Signal was scanned and quantified on Image Quant Las 4000 System.

### 2.6 Gas Chromatography Mass Spectrometry (GC/MS)

Supernatant fraction of urine samples, obtained as described above, was stored at -20°C until GC-MS. The day before measurement, samples were transferred at 4°C and maintained at this temperature overnight, in order to allow for slow defrosting. Four ml of urine supernatant were picked up, transferred in 20 ml Headspace-glass vial (SUPELCO, Bellefonte, PA, USA) and sealed using an aluminum crimp cap with PTFE/silica septum (SUPELCO, Bellefonte, PA, USA). Samples thus prepared, were kept at 4°C until VOC analysis. For each considered subject, two urine vials have been prepared: one for GC/MS and one for gas sensor array analyses. Urine samples were been taken out of the fridge

and left at room temperature for 5 minutes before GC/MS analysis. Then, vials were thermostated in water bath at 50°C for 10 minutes in order to reach VOC equilibrium in urine headspace, using the adjustable heater C-MAG HS 7 IKAMAG coupled to ETS-D5 thermometer (IKA®-Werke GmbH & CO. KG, Staufen, Germany). Adsorption of urine volatile compounds was carried out by Solid Phase Microextraction (SPME) technique. A 50/30  $\mu\text{m}$  divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen®/PDMS-SUPELCO, Bellefonte, PA, USA) fiber have been manually exposed to sample headspace for 1 hour at 50°C. Sampled volatile compounds thermally desorbed from the fiber at 250°C for 3 minutes, in the GC injection port. Chemical analysis were performed by means of GCMS-QP 2010 Shimadzu series gas chromatograph mass spectrometer (Shimadzu, Kyoto, Japan), equipped with EQUITY-5 (poly(5% diphenyl/95% dimethyl siloxane) phase, SUPELCO, Bellefonte, PA, USA) capillary column, 30m length  $\times$  0.25 mm I.D.  $\times$  0.25  $\mu\text{m}$  thickness. Analysis was performed in splitless mode using ultra-high purity helium as carrier gas. VOCs were separated on the GC column using an initial oven temperature of 40°C for 5 minutes, then increased by 5°C/min to 220°C, held for 2 min, after ramped to 250°C at 7°C/min and lastly at 20°C/min to 300°C that was held for 3min (total run time: about 48min).

The instrument has been controlled in linear velocity constant mode, using a carrier gas pressure of 24.9kPa, total flow of 5.9 ml/min, column flow of 0.7 ml/min and linear velocity of 30.2 cm/s.

The mass spectrometer was a single quadrupole analyzer operating in electron ionization mode, with an ionization energy of 70 eV, scanning over a mass range between 30 and 400m/z in full scan mode. The temperature of interface and ion source was kept constant at 250 °C.

The GC-MS data have been analyzed using the section GCMS post-run analysis of the GCMS solutions software (version 2.4, Shimadzu Corporation). Putative identification of compound was carried out by a comparison of mass spectra with both NIST 127 and NIST 147 libraries.

In order to avoid any contamination from previous uses, SPME fibers were conditioned before exposition to the first urine sample of the day, according to supplier guidelines.

## 2.7 Gas sensor array analysis

The urine samples have been maintained room temperature for 10 min before analysis, in order to achieve thermodynamic equilibrium in the headspace. Two needles are inserted into urine headspace through vial cap septum. One connected to the gas sensor array via a 30 cm tube PUN-03 (FESTO, IT), and used to uptake headspace portion, and the other to a calcium chloride dryer to avoid the formation of a vacuum in the vial during the measurements.

The instrument used for this study is the last version of gas sensor array developed at the University of Rome Tor Vergata. It consists of an ensemble of twelve quartz microbalances (QMBs), having a fundamental frequency of 20 MHz, functionalized using porphyrins and corroles. In low-perturbation regime, a mass change ( $\Delta m$ ) in sensing layer coating quartz surface results in a proportional frequency variation ( $\Delta f$ ). Sensor configuration used in this work is the adopted in a previous study on LOX-1 role in colorectal cancer prognosis [40].

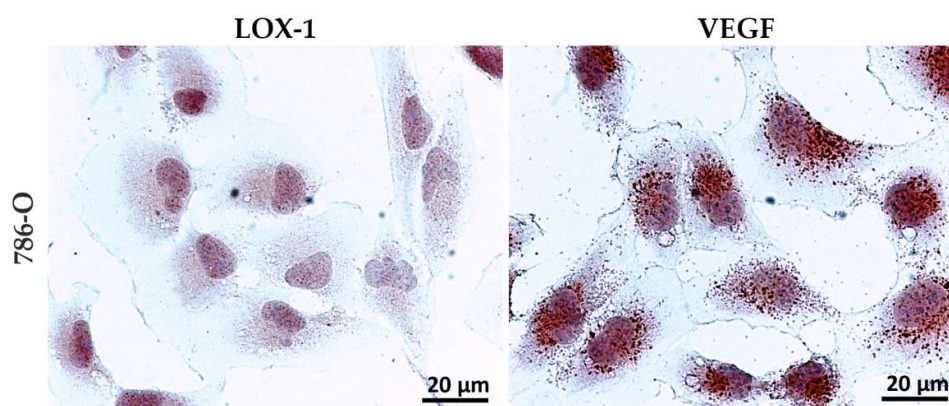
Sensor baseline signals have been obtained using a constant flow of 50 sccm (standard cubic centimetre per minute) of environmental air filtered by a  $\text{CaCl}_2$  trap. Sample headspace is analysed by the sensor array for 60 s. The difference between the oscillation frequency of the headspace sample and the reference air is the considered response of each QBM sensor, expressed in Hz.

Sensor system control and data acquisition are managed by an in-house software running in Matlab.

### 3. Results

#### 3.1 LOX-1 is expressed in a representative ccRCC cell line

First of all, we preliminary test LOX-1 expression in 786-O ccRCC cell line. As shown in Figure 1, LOX-1 expression is detected in 786-O by immunocytochemistry, evidencing a prevalent nuclear localization (++). In parallel, a similar expression pattern was observed for VEGF-A, modulated by LOX-1 according to literature data [40]. VEGF-A is expressed in a dotted fashion, mainly related to the nucleus (+/++), as compared to the cytoplasm (+).

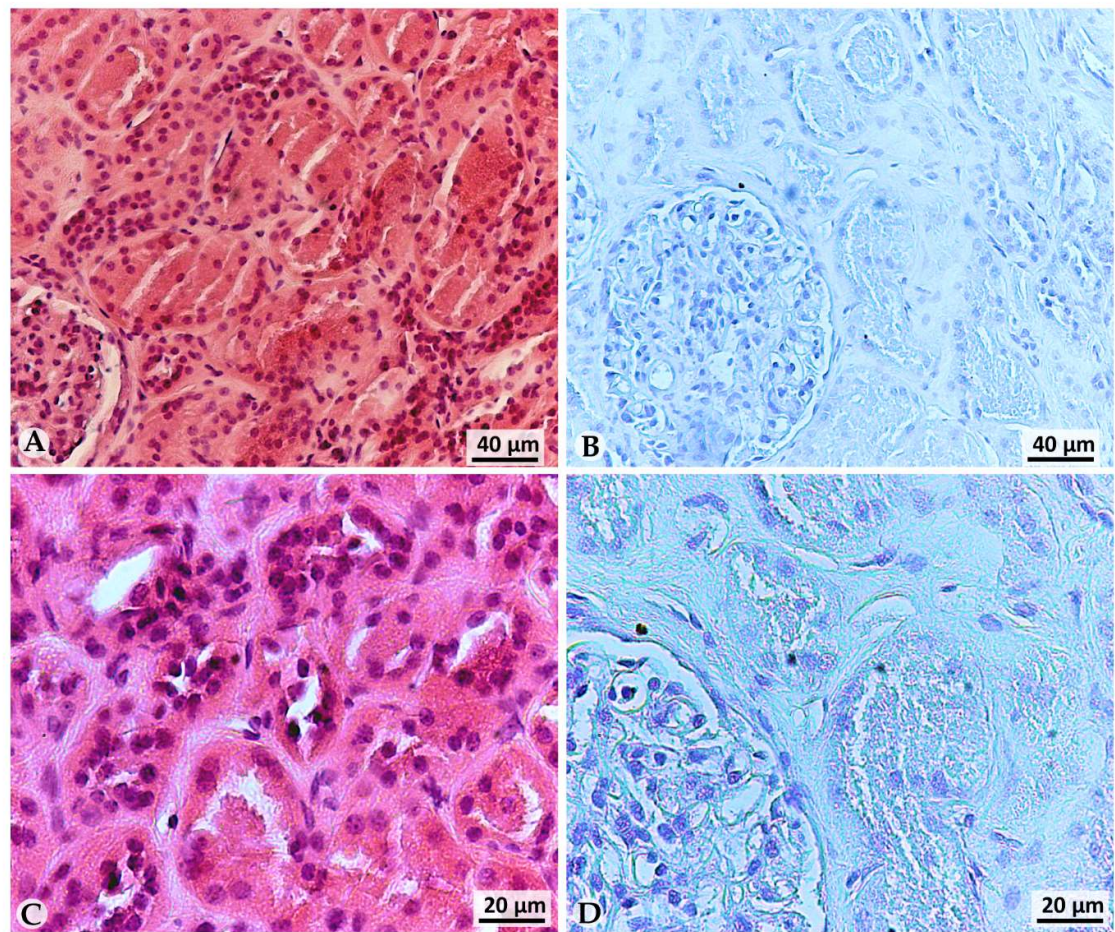


**Figure 1.** (A) LOX-1 and (B) VEGF-A immunocytochemistry analysis in 786-O ccRCC cell line. Scale bar 20um.

#### 3.2 LOX-1 is overexpressed in the extracellular space of G2 and G3 tumours

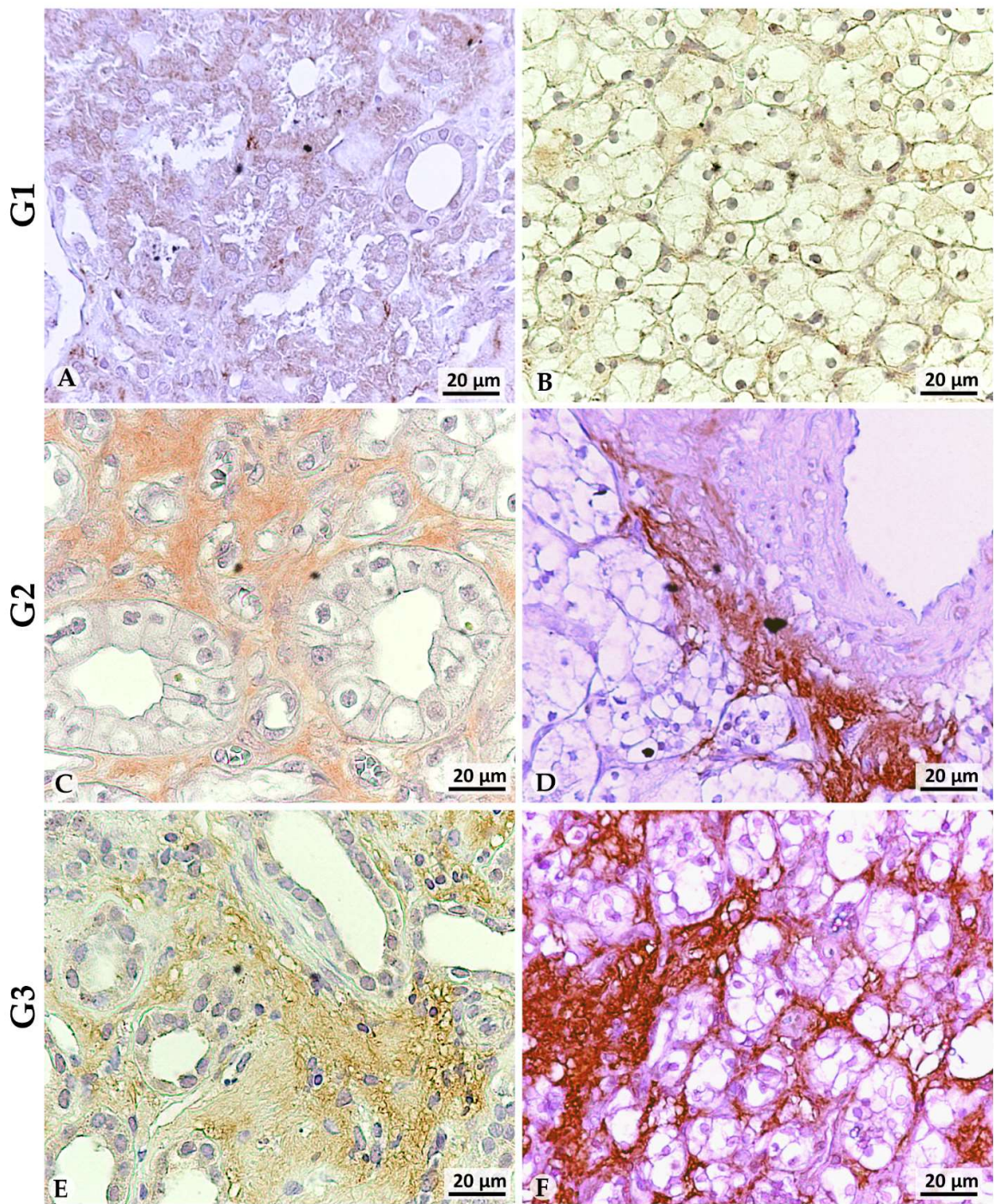
Tumoral tissue examination revealed kidney parenchyma with a focus of infiltrative clear cell population comprised of bland looking single cells or small cohesive nests, surrounded by rich capillary network. Tumoral cells contained slightly eccentric nuclei with inconspicuous nucleoli, visible only at high magnification, and a cytoplasmic glycogen storage.

In order to evaluate LOX-1 expression in clear cell renal carcinoma at different grade and stage, immunohistochemistry was performed on 40 human clear cell renal cancer tissues; healthy tissues aside the neoplasia (called Normal peritumoral Tissue; NpT) from the same patients were also examined. Additionally, eight autoptic healthy kidney tissues were examined for LOX-1 expression, as described in materials and methods. The histological features of the autoptic healthy kidney tissue are shown in Figure 2 A-C, in which haematoxylin/eosin staining allows to appreciate, at two different magnifications, the renal tubules. Immunohistochemical investigation showed that LOX-1 expression was completely absent in all autoptic healthy tissues analyzed (n=8) (Figures 2B-D).



**Figure 2.** Ematoxylin/Eosin staining and LOX-1 immunoistochemistry of healthy autopsy kidney tissue. (A-C) Ematoxylin/Eosin staining, (B-D) LOX-1 immunoistochemistry, (A-B) magnification 20x, (C-D) magnification 40x.

In contrast, differential LOX-1 expression was found in NpT tissues according to tumour grade. In NpT G1 tissues (n=8), a clear expression of LOX-1 was observed exclusively in the cytoplasm (++) (Figure 3A). Conversely, LOX-1 expression was detected both in the nucleus (+) and also weakly in the cytoplasm (+/-) in the tumour counterpart (Figure 3B, Table 1). In addition, a discrete expression of LOX-1 can also be observed in the extracellular space (++) (Table 1, Figure 3B). The pattern of LOX-1 expression found in NpT G1 tissues remains the same also in NpT G2 (n=23) (Figure 3C), as LOX-1 expression is related only to the cytoplasm, although less evident (+). This expression is different in the tumour counterpart: 12 of 23 G2 cases show only 10% of nuclei weakly positive for LOX-1 expression (+/-), while the signal is completely absent in the cytoplasm. In contrast, strong LOX-1 expression is observed in the extracellular space (++) (Figure 3D). Finally, in NpT aside G3 tissue (n=9), LOX-1 expression was detected again exclusively in the cytoplasm (+) (Figure 3E), while the tumour counterpart lost the weak nuclear positivity found in G2 and consequently showed a strong positivity in the stroma (++) suggesting that this protein may acquire an extracellular localization, being released from tumour cells (Figure 3F) (Table 1).

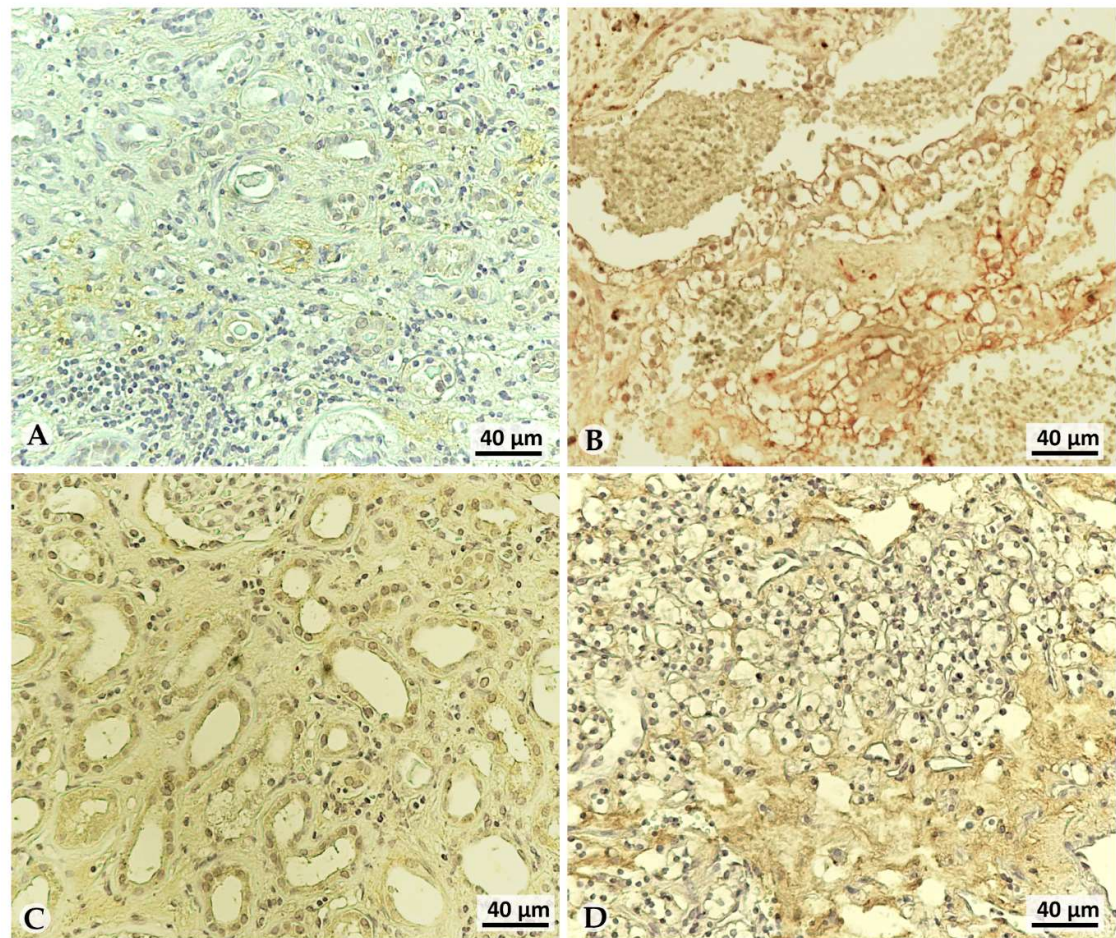


**Figure 3.** LOX-1 immunoistochemistry analysis in clear cells kidney cancer. (A) NpT aside G1 tumor, (B) G1 tumor, (C) NpT aside G2 tumor, (D) G2 tumor, (E) NpT aside G3 tumor, (F) G3 tumor. Scale bar 20μm.

**Table 1.** Distribution of LOX-1 in tumor and healthy counterpart of different grade and stage

Patients	Grade	Normal peritumoral tissues (NpT)		Tumoral tissues		
		Nucleus	Cytoplasm	Nucleus	Cytoplasm	Extracellular space
8	G1	-	++	+	+/-	50% ++
23	G2	-	+	10% cells +/-	-	++
9	G3	-	+	-	-	++

VEGF expression was barely detectable in NpT aside G2 tumor (Figure 4A), while in G2 tumour tissue results significantly detected, as shown in Figure 4B. LOX-1 expression correlates with VEGF-A in the corresponding tissue (Figure 4 C: NpT; Figure 4 D: tumor), suggesting the correlation between the two proteins.



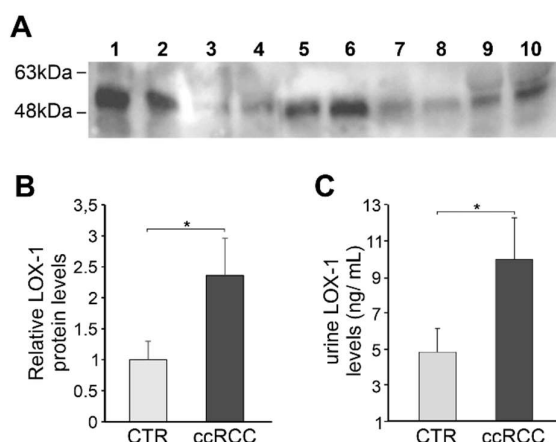
**Figure 4.** VEGF-A and LOX-1 immunohistochemistry analysis in NpT and tumoral tissues. (A-C) NpT tissues, (B-D) G2 tumoral tissue, (A-B) Immunohistochemistry of VEGF expression, (C-D) LOX-1 expression in high grade is present also in the extracellular space. Scale bar 40μm.

### 3.3 LOX-1 levels are impaired in clear cell renal cancer (ccRC) urine patients

With the aim of verifying whether LOX-1, that seems to be progressively accumulated in the extracellular area according to tumour grade, was finally released, we analyzed and quantified the protein in urine collected from ccRCC patients and healthy controls by Western blot and enzyme-linked immunosorbent assay (ELISA).

Firstly, we performed a Western blot to evaluate a possible presence and accumulation of LOX-1 protein in urine. The analysis has suggested that the expression of ox-LDL receptor protein was higher in urine of patients compared to control group (Figure 5A), as revealed by densitometric analysis on the mean value of tumors versus healthy controls (Figure 5B; p-value = 0.05).

In order to confirm more precisely this uncovering, we quantified the levels of LOX-1 in urine by ELISA, following procedures described in M&M. This analysis revealed a marked increase of LOX-1 levels in urine of patients (n=30) compared to control group (n=25) (Figure 5C). Although a discrete heterogeneity was present within groups, also in this case the data results to be statistically significant (p-value <0.05). Moreover the increasing trend of protein quantity results to be proportionally correlated to the tumoural grading.

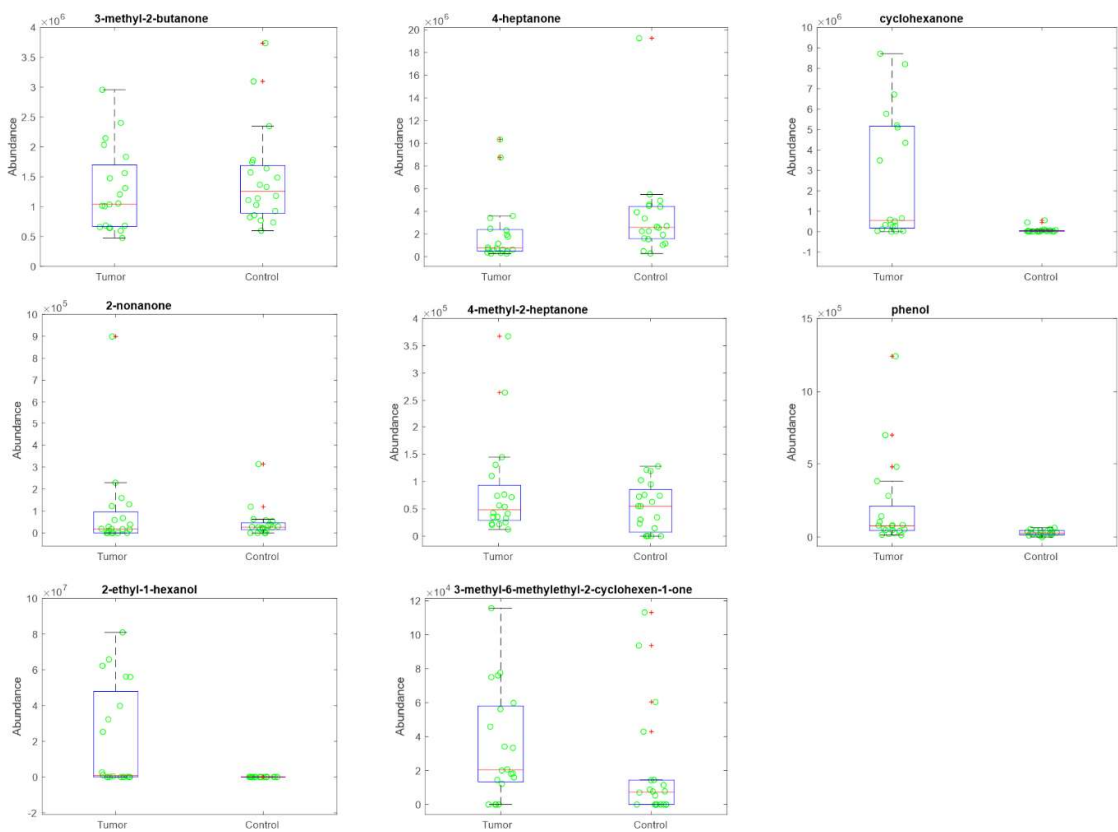


**Figure 5:** LOX-1 is revealed in urine of ccRC patients. **(A)** Representative Western blot analysis of equal amount of urine normalized with total protein from ccRCC patients (n=6; line:1-2-5-6-9-10) and controls (n=4; line:3-4-7-8). **(B)** Data are reported as mean  $\pm$ SEM (p-value =0.05) LOX-1 levels analyzed by ELISA in ccRCC urine samples (n=30) and healthy controls (n=25). **(C)** Data are reported as mean  $\pm$ SEM (p-value<0.05)

### 3.4 Volatile compounds analysis in urine

The volatile compounds released by urines have been analyzed with the purpose to determine a set of volatile compounds whose pattern can selectively discriminate kidney cancer samples from wild type ones.

Urines from 40 subjects have been collected and analyzed with gas chromatography mass spectrometer (GC/MS) and a gas sensor array in order to determine a set of volatile compounds whose pattern can selectively discriminate kidney cancer. In the totality of samples, GC/MS detected 98 volatile compounds. The majority of these compounds appeared only in some of the samples. Thus, in order to search VOCs univocally representative of the difference between cancer and control urines, the analysis has been limited to those compounds present in at least 70% of samples. These eight compounds, listed in Table 2, have been putatively identified by library comparison of mass spectra. The distribution of the abundance of each of these compounds in the categories of ccRCC kidney cancer and control is shown in figure 6. Kruskal-Wallis rank test was used to estimate the null-probability (p-value) of the difference between the two categories. P-value of each compound is shown in table 2. All these compounds have been previously found in human samples. The most discriminant compounds (4-heptanone, cyclohexanone, 1-hexanol-2-ethyl, and phenol) were previously indicated as potential biomarkers of kidney cancer [41,42]. In particular, cyclohexanone and 1-hexanol-2-ethyl are more abundant in the urines of cancer patients. Besides the relationship with kidney cancer, 3-methyl-butan-2-one and 4-heptanal are typical components of breath and urine [43], and the abundance of 2-nonanone in breath was found correlated with lung cancer [44].



**Figure 6:** Box plots of the abundances of the most recurrent volatile compounds respect to cancer and control groups.

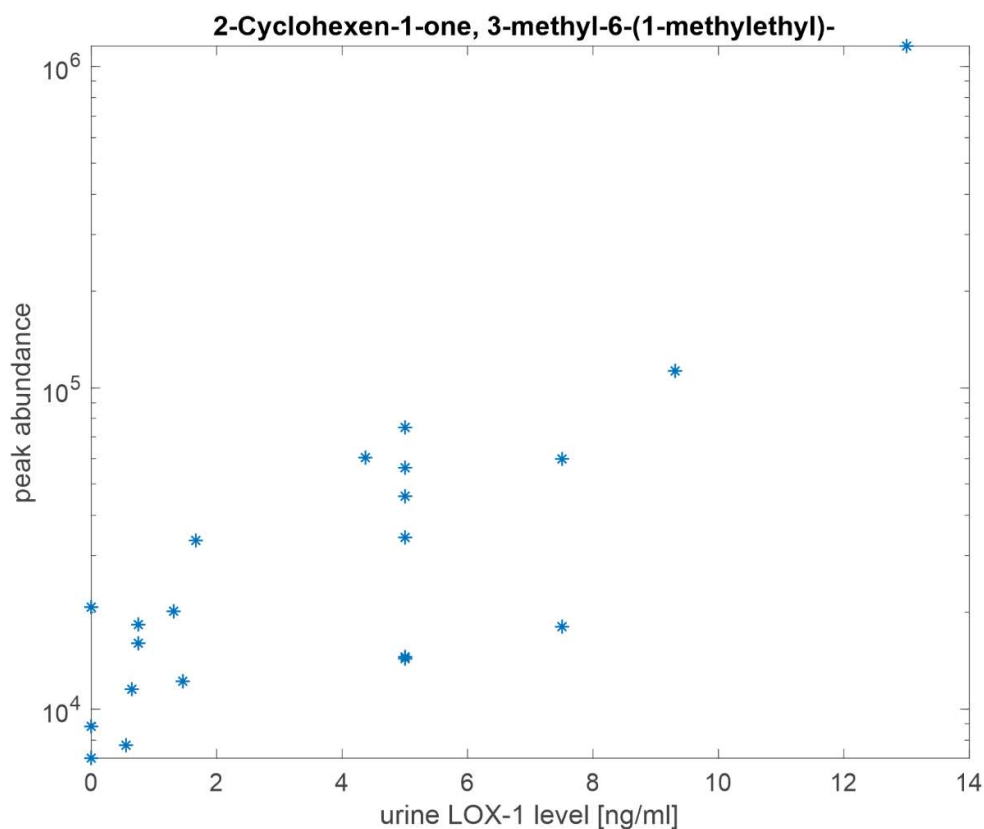
**Table 2.** Compounds present in at least 70% of samples

	VOC	p-value
1	2-Butanone, 3-methyl-	0.26
2	4-Heptanone	0.01
3	Cyclohexanone	<0.001
4	2-Nonanone	0.79
5	2-Heptanone, 4-methyl-	0.55
6	Phenol	<0.001
7	1-Hexanol, 2-ethyl-	<0.001
8	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)-	0.02

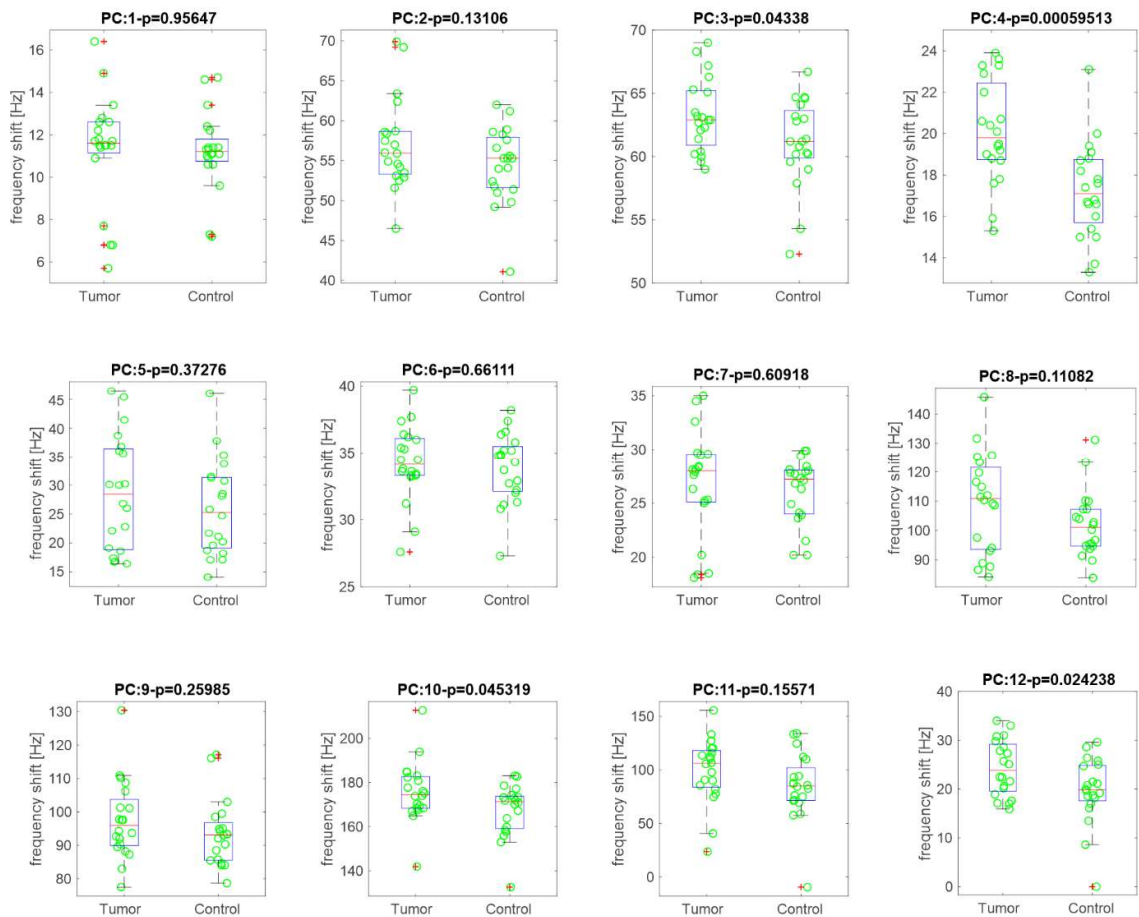
Finally, 2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)- has been indicated as a product of metabolism of ketones. In rats, it results the main metabolic product of (R)-5-Methyl-2-(1-methylethylidene) cyclohexanone, a monoterpene ketone that is main constituent of mint essential oil widely used as flavoring agent [45].

As shown in Figure 7, the logarithm of the abundance of 2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl) is linearly correlated with the concentration of LOX-1 in urines. Noteworthy, this is the only of the eight selected compound showing correlation with LOX-1 levels.

GC/MS analysis was complemented by a gas sensor array. Kruskal-Wallis rank test was applied to evaluate the capability of each sensor to discriminate between cancer and wild type urines. Figure 8 shows the box-plots of sensors responses, the title of each plot shows the corresponding p-value. Four sensors (labeled as 3, 4, 10, and 12) are characterized by  $p < 0.05$ .



**Figure 7:** the logarithm of the abundance of 2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)- respect the concentration of LOX-1 in urines.



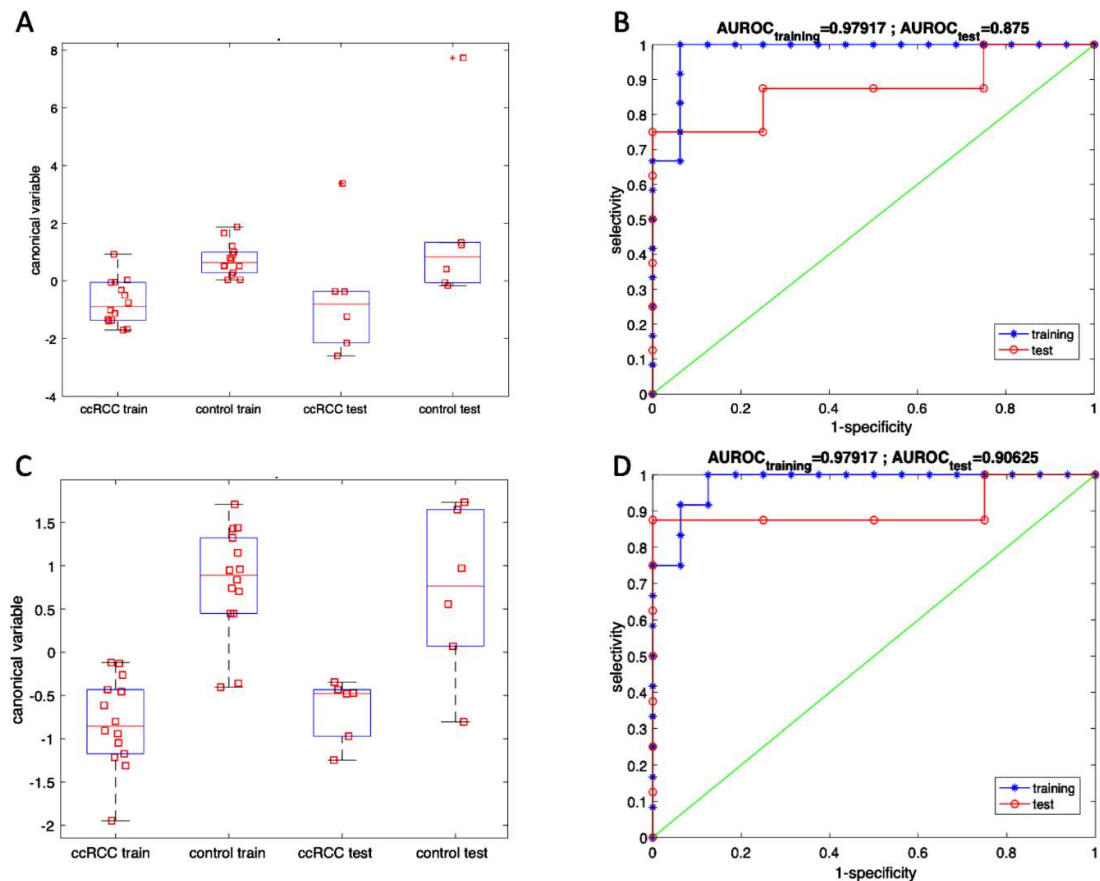
**Figure 8:** Box plots of sensor’s responses respect to cancer and control groups.

A better appraisal of the diagnostic properties of volatile compounds as detected by GC/MS and sensor arrays are achieved by classification algorithms. Here Linear Discriminant Analysis (LDA) has applied to GC/MS and sensors array data. The datasets have been split in two parts one used to train the model and the other to test. Random split of data in two groups may result in favourable conditions that could lead to optimistic conclusions. To avoid this drawback, LDA has been calculated 100 times, each time with a different partition of data in training and test. Accuracy, namely the percentage of correct classification of test data, has been considered as the indicator of the goodness of the model. Eventually, the LDA model corresponding to the average accuracy was retained as representative for the classification.

Figure 9 shows the canonical variables and the area under the ROC of both GC/MS and sensor array classification models while the results of classification are listed in Table 3.

**Table 3.** Distribution of LOX-1 in tumor and healthy counterpart of different grade and stage

	Accuracy		Sensitivity		selectivity	
	<i>Training</i>	<i>Test</i>	<i>Training</i>	<i>Test</i>	<i>Training</i>	<i>test</i>
GC/MS	92.9%	91.7%	85.7%	83.3%	100%	100%
Sensor array	92.9%	91.7%	100%	100%	85.7%	83.3%



**Figure 9:** Box-plots of canonical variable and ROC calculated with GC/MS and sensor array data respect to cancer and control groups. Canonical variables were calculated with training data and applied to test data.

#### 4. Discussion

RCC represents around 3% of all cancers, with the highest incidence occurring in Western countries [1,2]. During the last two decades until recently, there has been an annual increase of about 2% in incidence both worldwide and in Europe [1,2]. Verified risk factors for RCC include age, smoking, obesity, poorly controlled hypertension, diet, alcohol, environmental and genetic factors (i.e., VHL mutations) [4]. Unfortunately, the majority of RCC (over 63%) are diagnosed when the disease is locally advanced or at a metastatic stage and approximately two third of cases shows unfavorable prognostic factors (i.e., ISUP grade) at the diagnosis. In Europe, the 5-year overall survival for all types of RCC is 49% [7,8], which has recently showed important improvements mainly deriving from an increase in incidentally detected RCCs at earlier stages, better surgical techniques, and the availability of several targeted anticancer agents. Notably, diagnostic tools allowing an early diagnosis of RCC are unavailable in current clinical practice. Therefore, the search for reliable biomarkers for the early detection of RCC remains a major challenge. Although almost all studies on RCC tissue biomarkers have been highly promising, almost all of these are based on retrospective series with small sample size and relatively short follow-up [46]. The reliability of the assay used for marker detection represents another limitation of these studies [46]. Interestingly, the identification of molecular markers in body fluids (e.g., sera and urine), which can be used for screening, diagnosis, follow-

up, and monitoring of drug-based therapy in RCC patients, is one of the most ambitious challenges in oncologic research [46,47]. The global analysis of gene and protein expression profiles of biological specimens, like tissue, blood, or urine, is an emerging promising tool for new biomarker identification. The markers identified from this high throughput integrated “omics” technologies have promising potential but application in clinical diagnostics and practical improvement in disease management is not routine [48-50]. Blood and urine are an ideal source of biomarker, for theoretical, methodological, and practical reasons [51]. There have been some promising reports about potential biomarkers in sera, but the available data are insufficient to justify their routine clinical application [8]. Moreover, there is limited literature with regard to urine markers for RCC [8, 48]. This is even though, compared with most solid malignancies, urine might represent the body fluid in which a RCC biomarker can directly excreted and more easily detected to obtain an earlier diagnosis [48]. A rational approach toward the choice of reliable biomarker(s) aimed at an early diagnosis of malignancies is focusing the research on molecules linking various risk factors having a key role in the carcinogenesis of the disease.

*OLR1* (OMIM#602601) is a metabolic gene that encodes for the Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a chameleon major receptor, with a key role in the development of hypertension, diabetes mellitus, hyperlipidemia, obesity and its complications [21,31]. Several studies shed light on its role also in the stimulation of the expression of proangiogenic proteins, including NF- $\kappa$ B and VEGF [52].

To date, there is now no doubt on the function exerted by LOX-1 in tumor insurgence and progression and mostly in related neo-angiogenesis of different human cancers, such as glioblastoma, osteosarcoma, prostate, colon, breast, lung and pancreatic carcinoma [31]. The ox-LDL receptor is a fine-tuned interplay between lipid metabolic regulator, angiogenesis and EMT-inducing transcription factor, involved in the regulation of cancer metastasis, suggesting for the transmembrane receptor an interesting linking role between atherosclerosis, its various metabolic pathways and tumorigenesis [31].

In this study we evaluate and correlate the presence and the levels of LOX-1 protein in ccRCC patients at different tumor stage, in order to identify a new marker for early Kidney cancer detection by non invasive methods.

After assessing both LOX-1 and VEGF expression in vitro in a ccRCC cell lines in the cytoplasm as in the nucleus, we analyzed 40 human kidney tumors and 8 autoptic healthy kidney tissues. LOX-1 results to be completely absent in normal autoptic renal tissues, while a staining is present exclusively in the cytoplasm of NpT aside tumors of all grade. These data indicate that in normal kidney tissues its function is not requested, while normal peritumoral tissues (NpT) are probably influenced by soluble mediators and stress conditions originated from an uncontrolled growth of the tumoral mass, the same pathways that induce LOX-1 expression [53].

With regard to the tumour counterpart, in ccRCC at different grade and stage, a differential expression of LOX-1 correlated with tumor grade was found. Expression was both nuclear and weakly cytoplasmatic in G1, while G2 and G3 showed no expression in the nucleus or the cytoplasm. Interestingly, in all three grades, LOX-1 appears to be gradually released into the extracellular space to localize in the stroma, much more evidently in G2 and G3. The level of extracellular expression also seems to correlate with tumor grade.

In light of these data, we move to assess the presence of LOX-1 protein within urine, a peculiar circumstance still never described until now. Quantitative analysis performed by ELISA demonstrated higher LOX-1 levels in ccRCC urine that in control ones, in a statistically significant manner. Protein quantity is directly proportional to tumor grade. We hypothesized that one of the most likely carrier could be exosome, already involved in cancer signaling. Exosome are a subset of tiny extracellular vesicles manufactured by all cells and present in all body fluids. During cell growth, exosomes are actively produced and released in order to promote tumor growth, progression and metastatic spread [54]. More-

over it has been demonstrated that membrane cholesterol-lowering drugs induce membrane removal of LOX-1 surface receptor within exosome membranes [55]. This hypothesis needs further experiments to be sustained.

Cells that make up a tumor are highly metabolically active and these compounds arising from the tumor can easily cross from the cells into the urinary space, making this fluid ideal for metabolomic discovery of RCC biomarkers. Urine is an abundant biofluid that can be readily obtained by noninvasive means. Both aspects make urine a practical choice for developing a method of early diagnosis for renal cell carcinoma [56].

The analysis of volatile compounds is in agreement with previous findings. Although different instruments and methods were used, some of the biomarkers found by GC/MS data corresponds with those found in former researches [41,42].

GC/MS analysis have been carried out collecting VOCs onto SPME fiber. This is a solid phase where volatile compounds are accumulated in order to be released in the GC/MS. SPMEs are available in different materials which are oriented to maximize the accumulation of molecules with specified features. In this study, a general purpose SPME has been used. This is an optimal choice for untargeted metabolomics, however we cannot exclude that other SPMEs could provide additional information about the relationship between volatile compounds and kidney cancer.

Only one of the selected compounds has been found to be correlated with the concentration of LOX-1. Interestingly, this compound has been indicated as a product of the metabolism of ketones [45] and thus confirming its association with LOX-1 in turn linked to metabolism.

On the contrary, the lack of correlation of the other volatile biomarkers with LOX-1 indicate the existence of additional pathways for these compounds. Importantly, it also indicates that LOX-1 and VOCs provide independent informations that can be used to improve the diagnosis of kidney cancer.

The same samples analyzed by the GC/MS have also been measured with a gas sensor array. These instruments, also known as electronic noses, have been frequently used to classify samples characterized by a complex chemical composition such as biological samples. The results of electronic noses are limited to the classification, so they do not provide information neither about the composition nor about the abundance of compounds. Nonetheless, for their simplicity and relative low-cost they have been indicated for routine analysis. Results show that in terms of identification of kidney cancer respect to controls, GC/MS and electronic nose gave comparable results. So it may be concluded that electronic noses could be used as a diagnostic tool for kidney cancer. Of course, these are the results obtained with this particular dataset.

A manifold of sources contributes to urines composition, food, lifestyle, and drug uptake are expected to determine the chemistry of urines. Thus, environmental influences, repeatability and long-time performance of the instrumentation need to be verified in multicentric studies. Furthermore, studies with larger sample sizes are necessary to elucidate if and how co-morbidities influence the urine volatility.

## 5. Conclusions

Dosing LOX-1 in urine represents a novel, cost-effective, sensitive, fast and reliable strategy for RCC diagnosis based on an ultra-sensitive and ultra-selective noninvasive tool that, in combination with other tool as VOCs, could transform clinical management by enabling early detection of RCC and reducing unnecessary kidney biopsies and nephrectomies [57].

In the end, given the complexity of cancer treatment, it will likely require a combination of clinical and biologic approaches to fully realize the potential of precision oncology.

**Author Contributions:** MM, SP designed the study and write the first draft of the manuscript; MM, SP, RC, CDN, CG, CP, VP, YKM performed the experiments, collected and analyzed the data; AM, AO, MC, GS, GS, AM provided biological tissues and clinical information; FS, CDN, FT, GN, MR critically revised the manuscript; All authors read and approved the final submitted manuscript.

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