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Evaluation of urinary and serum level of chemokine (C-C motif) ligand 2 (CCL2) as a potential biomarker in canine urogenital tumours

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Introduction

Bladder transitional cell carcinoma represents approximately 2% of all tumours affecting dogs.[1] Metastases have been reported in 16% of dogs at the time of diagnosis and distant metastases were found in 58% of dogs at necropsy.[2] Prostatic tumours are mainly undifferentiated or transitional cell carcinoma with more than half with a mixed pattern including urothelial, glandular, squamous and sarcomatoid origins.[3,4] Prostatic carcinoma is highly metastatic with 80% dogs diagnosed with metastasis at necropsy. The treatment of urogenital carcinoma (UGCa) in dogs can be challenging due to a late diagnosis contributing to an extensive local invasion at the time of diagnosis.

There is sufficient proof that chronic inflammation predisposes individuals to development of various types of cancer, defining inflammation as a hallmark of cancer.[5,6] An increase in inflammatory cells and soluble inflammatory mediators, such as cytokines and chemokines in the primary tumour are mostly associated with poor prognosis due to metastasis. However, the immune system has a dual role in cancer progression that depends on the cellular context of infiltrating cells and the tumour type. Chemokines and chemokine receptors have a key role in cancer-associated inflammation, where the chemokine-chemokine receptor axis affects both the composition and the function of cells within the tumour microenvironment.

In humans, several chemokines such as CXCL1, CXCL2, CXCL12, have been reported to play a role in the development of the primary tumour or metastasis of urogenital tumours. Chemokine (C-C motif) ligand 2 CCL2 (previously named monocyte chemoattractant protein-1 or MCP-1) has been demonstrated to be highly

expressed by tumour cells in different cancer types in humans.[7-12] CCL2 serum and urinary level are increased in bladder and prostatic cancer in human patients.[13] Roles of this chemokine include local tumour invasion, cell migration, tumour cell intravasation and extravasation.[14] By attracting tumour-associated macrophages, CCL2 promotes angiogenesis via the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF),[15] epidermal growth factor (EGF),[16] and basic fibroblast growth factor (FGF-2). [17,18] Moreover, when binding to its receptor CCR2 at the surface of endothelial cells, CCL2 induces cytoskeletal retraction within the vascular endothelial cells, forming a gap between cells and allowing transmigration of tumour cells into the bloodstream.[19] CCL2 is related to an increased migration activity of the tumour cells through the activation of protein kinase C and phosphorylation of paxillin. [12] The significance of the role of CCL2/CCR2 in the pathogenesis of bladder cancer is emphasized by the fact that humans with specific polymorphisms in the CCL2 and CCR2 genes are at increased risk of developing bladder cancer.[20] In human patients with urinary bladder carcinoma, urinary levels of CCL2 have been demonstrated to correlate significantly with stage and grade of the tumour and may be a prognostic marker for the natural course of the disease.[10]

CCL2 has been studied sparsely in dogs with cancer. In a retrospective study involving dogs with lymphoma, it was shown that peripheral neutrophil and monocyte counts as well as serum CCL2 concentrations were significantly elevated relative to healthy control animals, and that such increases were associated with a decreased disease-free interval in dogs treated with chemotherapy.[21]

However, no study has evaluated CCL2 level in canine UGCa. Therefore, the aim of the study was to investigate urinary and serum CCL2 levels as potential biomarkers in canine UGCa. We hypothesized that CCL2 levels in serum and urine would be higher in dogs with UGCa compared to healthy animals and that dogs with metastasised UGCa would have a higher CCL2 concentration compared to the non-metastasised animals.

Materials and Methods

Dog population

Dogs were classified into six different groups: healthy, bacterial cystitis, bacterial prostatitis, benign prostatic hyperplasia (BPH), UGCa without evidence of metastasis and UGCa with evidence of metastasis.

The healthy group included 10 male and 10 female Beagle dogs with no clinical signs of urinary tract disease, no current medications and with normal blood test (biochemistry and haematology) and urinalysis (urinary dipstick, urine specific gravity, and cytology). Dogs were born and housed in XXX (XXX). Animal housing, care, and experimental procedures were approved by the Ethical Committee of Animal Use of the University of XXX (reference n°1630).

Dogs from the other groups were presented at our hospital and diagnostic tests were performed at the university veterinary hospital (XXX, XXX). Blood and urine were sampled after owner consent.

Bacterial cystitis, prostatitis and BPH were confirmed with ultrasound, urinary or prostatic wash culture and cytology. Dogs under antibiotic or anti-inflammatory medication were not included in these groups. Bladder carcinoma was confirmed by

histology from an ultrasound-guided biopsy,[22] or cytology from a brush biopsy. Prostatic carcinoma was diagnosed by cytology from ultrasound-guided fine-needle aspirates. Dogs with UGCa underwent complete staging including abdominal ultrasound, fine-needle aspiration and regional lymph node cytology, and thoracic radiographs. Dogs under any treatment such as chemotherapy or anti-COX-2 medication were excluded from the UGCa groups.

Sample collection and processing

Blood was collected by venipuncture at the jugular vein. Soon after collection, blood samples were centrifuged at 4°C for 15 min at 1300g and serum was aliquoted into plastic cryotubes. Urine was collected in an aseptic manner by catheterisation in dogs with UGCa or by cystocentesis in healthy dogs and in dogs with other urinary tract disease. Urine samples were centrifuged at 4°C for 10 min at 700g and for 20 min at 1300g. Cell-free urine was then aliquoted into plastic cryotubes. Serum and urine aliquots were frozen within 30 min of collection and stored at -80°C until measurement.

Immunoassay

CCL2 concentration measurements were performed with a commercially available immunoassay kit (Quantikine ELISA canine MCP-1, R&D Systems) on non-diluted serum and on 1:8 diluted urine samples, in duplicate, following the manufacturer's instructions. Urine samples had also creatinine concentration measured by the Jaffé method with an automated analyser (Respons 920, Diasys). CCL2 concentrations were expressed in pg/ml for serum samples and as a CCL2 : creatinine ratio for urine samples.

Statistical analysis

Prior to the study, power analysis was calculated based on previous results of Perry *et al.*[21] Using this standard deviation with an alpha of 0.05, a power of 93% could be reached with a number of 10 dogs per group. Normality of data was assessed with the Kolmogorov-Smirnov test. Multivariate analysis was performed using a linear model including regression on weight, age, sex and disease status. The model was as follow: $y_{i,j} = \mu + \beta * a_{i,j,k} + \gamma * w_{i,j,k} + s_i + d_j + (sd)_{ij} + e_{ij}$ (where 'a' is the age, 'w' is the weight, 's' is the sex, 'd' is the disease status and 'sd' is the interaction between sex and disease status). Univariate analysis was performed with a Mann-Whitney test. P-values less than 0.05 were considered to be significant. A commercially available software package (SPSS 24.0®) was used for data analysis.

Receiver Operating Characteristic (ROC) curves as well as calculation of the Area Under the Curve (AUC) were made with commercially available MedCalc 16.4® Software. The test accuracy was classified as excellent (if AUC>0.9), good (0.8<AUC<0.9), fair (0.7<AUC<0.8), poor (0.6<AUC<0.7) or failed (0.5<AUC<0.6). The optimal cut-off values were determined by the Youden index method.[23]

Based on the prevalence of UGCa and its metastases in dogs with signs of urinary tract disease in our practice, the positive and negative predictive value (PPV and NPV respectively) were calculated. Positive predictive value was defined as the proportion of positive results that are truly positive results. Negative predictive value was defined as the proportion of negative results that are truly negative results.

Results

Seventy-nine dogs were included in the study. Mean age was 8.1 years (range: 5 months to 15.3 years). Mean body weight was 16 kg (range: 4.8 kg to 53 kg). The age of healthy dogs was comparable to diseased dogs.

Ten female and 10 male Beagle dogs were included in the healthy group. Ten female and 6 male dogs were included in the bacterial cystitis group. Ten male dogs were included in the bacterial prostatitis group and 10 male dogs in the BPH group. Twenty-three dogs with UGCa were included, 7 female and 7 male dogs in the metastasised UGCa group, and 9 female dogs in the non-metastasised UGCa group. No male was included in the non-metastasised UGCa group.

Validation of a canine CCL2 ELISA kit for urine samples

A linearity/recovery test was performed on urine samples. In our selected samples, recovery was 98% for the UGCa sample, 120% for the cystitis sample and 114% for the healthy sample. Linearity ranged between 90 and 113%. These results allowed validating the use of this ELISA kit on canine urine samples.

CCL2 concentration in serum

No significant influence of age, weight and gender was detected on CCL2 concentration in serum in all groups.

There was a significant difference in CCL2 concentration between healthy dogs and all urogenital tract diseased dogs (including inflammatory and neoplastic state) (P=0.015), and between healthy dogs and dogs with UGCa (metastasised or not) (P<0.01) with a median value of 141.9 pg/ml (range=[69.8-457.3]) for healthy dogs, a median value of 205.8 pg/ml (range=[41.2-1841.6]) for all urogenital tract diseased dogs (including both inflammatory and neoplastic state), and a median value of 421.9

pg/ml (range=[104.3-1841.6]) for dogs with UGCa (metastasised or not). Dogs with non-neoplastic urogenital tract disease had a significantly lower serum CCL2 concentration (median=164.3 pg/ml; range=[41.2-1251.8]) than dogs with UGCa (median=421.9 pg/ml, range=[104.3-1841.6]).

There was no significant difference in serum CCL2 concentration between the bacterial cystitis, bacterial prostatitis and the BPH group with a respective median concentration of 176 pg/ml (range=[104.1-1251.8]), 141.7 pg/ml (range=[41.2-645.3]) and 145.7 pg/ml (range=[52.6-291.5]).

The metastasised UGCa group had a significantly lower CCL2 concentration (median=296.2 pg/ml; range=[104.3-1259.2]) compared to the non-metastasised UGCa group (median=903.2 pg/ml; range= [194.7-1841.6]) (P<0.01) (Figure 1).

CCL2 : creatinine ratio in urine

No significant influence of age, weight and gender was detected on CCL2 : creatinine ratio in urine in all groups.

There was a significant difference between the urine CCL2 : creatinine ratio in healthy dogs and all urogenital tract diseased dogs (including inflammatory and neoplastic state) (P<0.001) and between healthy dogs and dogs with UGCa (metastasised or not) (P<0.01) with a median ratio of 2.9 x 10^7 (range=[0.87 x 10^7 - 31.9 x 10^7]) for healthy dogs, a median ratio of 20.7 x 10^7 (range=[0.9 x 10^7 - 1162.0 x 10^7] for all urogenital tract diseased dogs (including both inflammatory and neoplastic state), and a median ratio of 22.2 x 10^7 (range=[4.3 x 10^7 - 282.9 x 10^7]) for all dogs with UGCa.

No significant difference was found in urinary CCL2 : creatinine ratio between dogs with non-neoplastic urogenital disease and dogs with UGCa (P=0.115).

There was also no significant difference (P=0.493) in urinary CCL2 : creatinine ratio between the bacterial cystitis, bacterial prostatitis and the BPH group with a respective median ratio of 27.1 x 10^7 (range=[0.9 x 10^7 - 1162.0 x10^7]), 20.7 x 10^7 (range=[2.0 x 10^7 - 90.8 x 10^7]) and 11.3 x 10^7 (range= [2.3 x 10^7 - 106.1 x 10^7]).

The metastasised UGCa group had a significantly lower urine CCL2 : creatinine ratio (median=20.0 x 10^7, range=[4.3 x 10^7 - 282.9 x 10^7]) compared to the non-metastasised UGCa group (median= 97.3 x 10^7, range=[15.8 x 10^7 - 253.5 x 10^7] (P<0.01) (Figure 2).

There was a good correlation (r= 0.73; P<0.01) between the serum CCL2 concentration and the urine CCL2 : creatinine ratio (Figure 3).

CCL2 as a diagnostic marker in the serum

ROC curves were calculated. To differentiate between dogs with UGCa and dogs with a urogenital inflammatory state, the measurement of CCL2 concentration in the serum was fairly accurate (AUC=0.77; $CI_{95\%}$=[0.64-0.87]; P<0.0001) (Figure 4a).

Based on the ROC curves, a cut-off value for serum CCL2 concentration for detecting UGCa of 294.74 pg/ml was selected. This value was associated with a sensitivity of 65.2% and a specificity of 80.6%.

Using this cut-off value of 294.74 pg/ml in the serum to detect UGCa and based on the prevalence of UGCa, this test would lead to a PPV of 10.3% and a NPV of 90.4%.

CCL2 : creatinine ratio as a diagnostic marker in the urine

ROC curves were calculated. To differentiate between dogs with UGCa and dogs with an urogenital inflammatory state, CCL2 : creatinine ratio in urine was fairly accurate (AUC=0.63; $CI_{95\%}$=[0.49-0.75] ; P=0.09) (Figure 4b).

Based on the ROC curves, a cut-off value for CCL2 : creatinine ratios for detecting UGCa of 8.52 x 10^7 was selected. This value was associated with a sensitivity of 95.2% and a specificity of 38.2%.

Using this cut-off value of 8.52 x 10^7 in the urine to detect UGCa and the prevalence of UGCa, this test would lead to a PPV of 3% and a NPV of 99.7%.

CCL2 as a staging marker in the serum

ROC curves were calculated. To differentiate between UGCa dogs with or without metastasis, the measurement of serum CCL2 showed a good accuracy (AUC= 0.84; $CI_{95\%}$=[0.63-0.96], *P*=0.0001) (Figure 5a).

Based on the ROC curve, a cut-off value for serum CCL2 concentration for detecting UGCa metastases of 421.9 pg/ml was selected. This was associated with a sensitivity of 88.9% and a specificity of 78.6%.

In our cohort of dogs, the prevalence of metastasis in dogs with UGCa was 60.9%. Using this prevalence and the cut-off value of 421.9 pg/ml, the serum CCL2 concentration would have a PPV to detect metastasis of 86.6% and a NPV of 81.9%.

CCL2 : creatinine ratio as a staging marker in the urine

ROC curves were calculated. To differentiate between UGCa dogs with or without metastasis, the urine CCL2 : creatinine ratio showed a good accuracy (AUC=0.71 ; $CI_{95\%}$=[0.48-0.89], *P*=0.0831) (Figure 5b).

Based on the ROC curves, a cut-off value for urine CCL2 : creatinine ratios for detecting UGCa metastasis of 35.95 x 10^7 was selected. This was associated with the absence of metastasis with a sensitivity of 85.7% and a specificity of 57.1%.

In our cohort of dogs, the prevalence of metastasis in dogs with UGCa was 60.9%. Using this prevalence and the cut-off value of 35.95 x 10^7, the urine CCL2 : creatinine ratio would have a PPV to detect metastasis of 75.7% and a NPV of 71.9%.

Discussion

In this study, we evaluated CCL2 concentration in the serum and urine of healthy dogs, dogs with urogenital inflammatory state and dogs with UGCa. CCL2 was elevated in serum and urine samples of dogs with UGCa compared to healthy dogs. CCL2 concentration was higher in neoplastic dogs and this concentration was further increased in the absence of metastasis. The accuracy of the measurement of CCL2 concentration as a diagnostic tool both in serum and urine is fair; however, its accuracy in differentiating metastatic and non-metastatic disease is good. The exact mechanisms of this CCL2 increase in neoplasia and inflammatory state is yet unknown. Some reports suggest that CCL2 mobilisation is mediated by inflammatory cytokines such as TNF and interleukins in humans.[24,25] CCL2 increases in dogs with neoplasia could therefore be linked to the tumour inflammatory microenvironment.

In our study, age and weight did not influence CCL2 levels neither in the serum nor in the urine samples. This result contrasts with human literature where serum CCL2 concentration increased with age in healthy patients.[26] The reason of this age dependant CCL2 level is unknown. It has been suggested that higher CCL2 concentration in elderly people may be due to a subclinical atherosclerosis or an age-dependant shift between T-helper cell dependent cytokine patterns.[26] The difference between our study and the latter may be due to species specificity. Extensive local invasion in bladder or prostatic carcinoma is often observed due to late diagnosis. In humans, invasive work-up (such as cystoscopy or biopsies) is needed in order to

detect local recurrence. This has led to the search for biomarkers helping in the early detection of the disease or its recurrence. In humans, four urinary biomarkers have been validated for bladder carcinoma: the nuclear matrix protein 22 (NMP-22; Se= 61.8%, Sp=80.3%), the bladder tumour antigen (BTA ; Se=64%, Sp=76%) , the cytokeratin-based test urinary bladder carcinoma antigen (UBC ; Se=64.4%, Sp=80.3%) and the Cyfra 21-1 (Se=64.4%, Sp=85.5%).[27] All these markers have a higher sensitivity than urine cytology in humans. However, the sensitivity stays lower than the cystoscopy, which is considered in human medicine as a standard to detect recurrence of bladder tumour. In veterinary medicine, the search for biomarkers has also gained popularity. Cytology, as in human medicine, has a low sensitivity and specificity. BRAF V595E mutation in the urine detected with digital droplet PCR (ddPCR) has been described.[26] The V595E mutation has been reported in 87% of UGCa. The use of ddPCR has shown an 85% sensitivity and an 100% specificity in free catch urine.[28] In canine urothelial carcinoma, it has also been reported that three regions of the canine genome were subject to recurrent copy number changes. These copy number imbalances were detected in 67% of urine samples.[29] A multiplex biomarker approach suggested four proteins as potential biomarkers (macrophage capping protein, PRX1, PRX6 and ribonucleoprotein A2/B1) but further studies would be needed to assess their sensitivity and specificity.[30] The BTA test validated in humans showed in canine samples (v-BTA) a high false positive rate with a PPV of only 2.8% and a NPV of 100%.[31] Detection of urine canine calgranulin $S100A_8/A_{9USG}$ showed a PPV of 3% and a NPV of 100% (Se= 86% and Sp= 80%). In our study, serum CCL2 or urinary CCL2 : creatinine ratio as diagnostic biomarkers showed a similar trend with nearly 100% of dogs with a negative test having no carcinoma (NPV=90.4% and 99.7%, respectively), while the PPV was only 10.3% and 3%,

respectively. As such, the use of CCL2 level both in serum and urine as a sole diagnostic test would not be indicated for detecting canine UGCa but could help to exclude UGCa. In humans, the use of urine CCL2 level as a sole diagnostic test had a 14.8% sensitivity and a 96% specificity. However, a combined assay with NMP-22, urinary intercellular adhesion molecule, CCL2 and cytology had a 80% sensitivity and a 100% specificity in muscle invasive bladder tumours (PPV=100%, NPV=99.6%).[11] It could be interesting to combine different molecular tests in dogs to know if this would increase the accuracy and if this could be used in a clinical setting.

Previous biomarkers reported in humans and canine UGCa are overexpressed when grade or stage is elevated. Increased CCL2 level has been correlated with tumour stage and grade in human bladder carcinoma.[7,10,15] Its increased level is also associated with a worse overall prognosis in prostatic carcinoma in humans.[32] In *in vitro* studies and in mouse models, CCL2 has been reported to facilitate metastasis in different cancer type in humans.[33,34,35] Interestingly, in our study, we found that a low level of CCL2 was associated with metastasis and that the use of CCL2 level in serum and urine lead to a good PPV and NPV. Serum CCL2 test had a PPV of 86.6% and NPV of 81.9%. Urine CCL2 : creatinine ratio had a PPV of 75.5% and NPV of 71.9%. The role of CCL2 in tumorigenesis in unclear. Overexpression of CCL2 in murine colon carcinoma showed a suppression of metastatic process[36] and high level of CCL2 has been reported to be a favourable prognostic factor for pancreatic carcinoma in humans.[37] *In vitro* and *in vivo* studies described a dual role of CCL2 in tumorigenesis in melanoma cells and pancreatic cells.[38,39] CCL2 can recruit monocytes and the effect of monocyte in tumour growth depends on their level of infiltrate. When low concentrations of CCL2 (0.5-5 PFU/cell) was added to tumour cells, neovascularisation and tumour growth was observed. However, at a higher dose

(50 PFU/cell), no vascularisation was observed but rapid necrosis was seen secondary to a massive infiltration of mononuclear cells into the tumour.[39] The use of an antibody anti-CCL2 on tumour cells inhibits the recruitment of intra-tumoral monocytes but not the peri-tumoral monocytes/macrophages, leading *in vivo* to a four-fold increase in tumour growth.[39]

In human pancreatic carcinoma, high levels of serum CCL2 were associated with a low Ki-67. High levels of serum CCL2 were also correlated with an increased iNOS concentration among tumour cell cultures generating NO, thus inducing G1 phase arrest and apoptosis.[37] Monti *et al.* suggested that CCL2 may not be indispensable but may favour tumour death by two possible mechanisms: massive monocyte/macrophage recruitment into the tumour, and/or an increased NO production inducing apoptosis.[37]

Our study aim was to assess if CCL2 would be a good biomarker for UGCa in dogs. It would be interesting to correlate CCL2 level with macrophages invasion and to investigate potential factors which may influence CCL2 production and whether this may enhance or inhibit tumour progression in canine UGCa.

In conclusion, the results of this study indicate that CCL2 in serum and urine as sole diagnostic tool could not be used to differentiate UGCa from other urogenital diseases in dogs but could help in excluding UGCa. CCL2 level in serum and urine may also be of importance during staging or as a prognostic marker. In this study, a low level of CCL2 was associated with metastasis in dogs with UGCa. However, further investigation is needed to confirm these findings and further studies are needed to investigate the role of CCL2 in canine urogenital tumours.

Conflict of interest statement

The authors declare no conflict of interest

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Figures legends

Figure 1: Box plot graph representing serum CCL2 concentration in healthy dogs, dogs with bacterial cystitis, bacterial prostatitis, benign prostatic hyperplasia, metastasised and non-metastasised urogenital carcinoma. *Median concentration, minimum, maximum, first and third quartile are represented.*

Figure 2: Box plot graph representing urine CCL2 : creatinine ratio in healthy dogs, dogs with bacterial cystitis, bacterial prostatitis, benign prostatic hyperplasia, metastasised and non-metastasised urogenital carcinoma. *Median concentration, minimum, maximum, first and third quartile are represented.*

Figure 3: Correlation curve and trendline between serum CCL2 concentration and urine CCL2 : creatinine ratio *($r= 0.73$; $P<0.01$).*

Figure 4 : ROC curve and AUC to differentiate between dogs with a urogenital carcinoma and dogs with inflammatory state using CCL2 level in the serum (a) and in the urine (b). *Youden index cut-off with their associated sensitivity (Se) and specificity (Sp) are presented for each curve*.

Figure 5 : ROC curve and AUC to differentiate between dogs with a urogenital carcinoma with metastasis and without metastasis using CCL2 level in the serum (a) and in the urine (b). *Youden index cut-off with their associated sensitivity (Se) and specificity (Sp) are presented for each curve.*

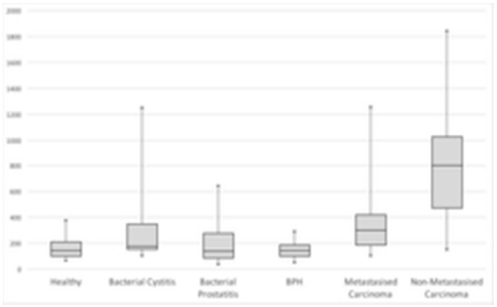


Figure 1: Box plot graph representing serum CCL2 concentration in healthy dogs, dogs with bacterial cystitis, bacterial prostatitis, benign prostatic hyperplasia, metastasised and non-metastasised urogenital carcinoma. Median concentration, minimum, maximum, first and third quartile are represented.

20x12mm (300 x 300 DPI)

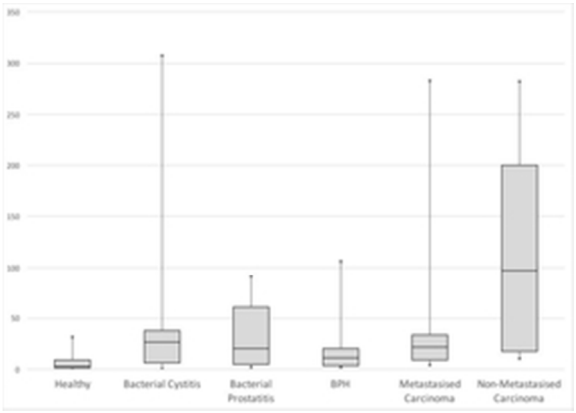


Figure 2: Box plot graph representing urine CCL2 : creatinine ratio in healthy dogs, dogs with bacterial cystitis, bacterial prostatitis, benign prostatic hyperplasia, metastasised and non-metastasised urogenital carcinoma. Median concentration, minimum, maximum, first and third quartile are represented.

24x17mm (300 x 300 DPI)

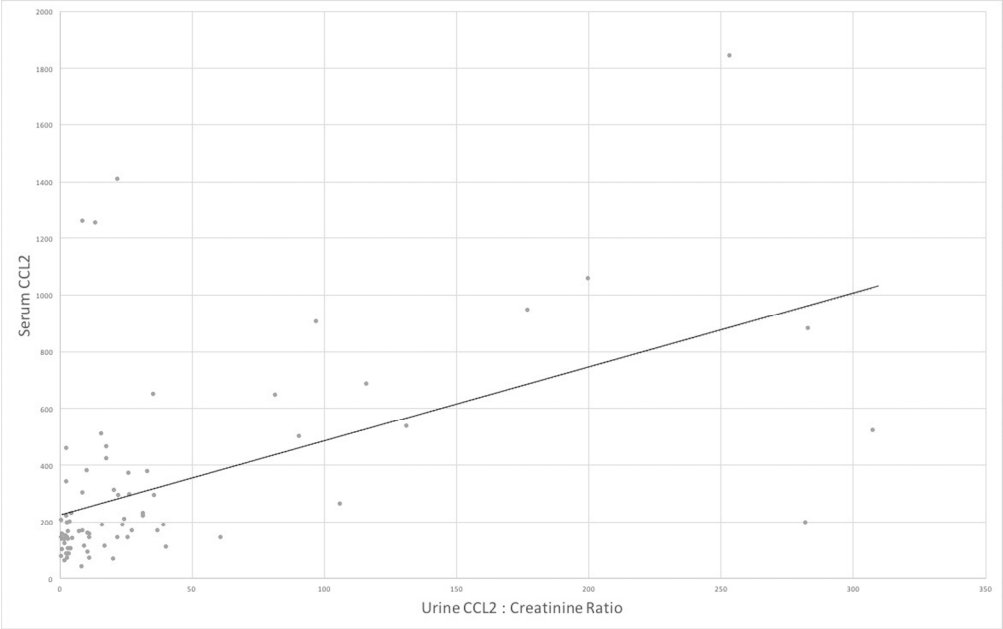
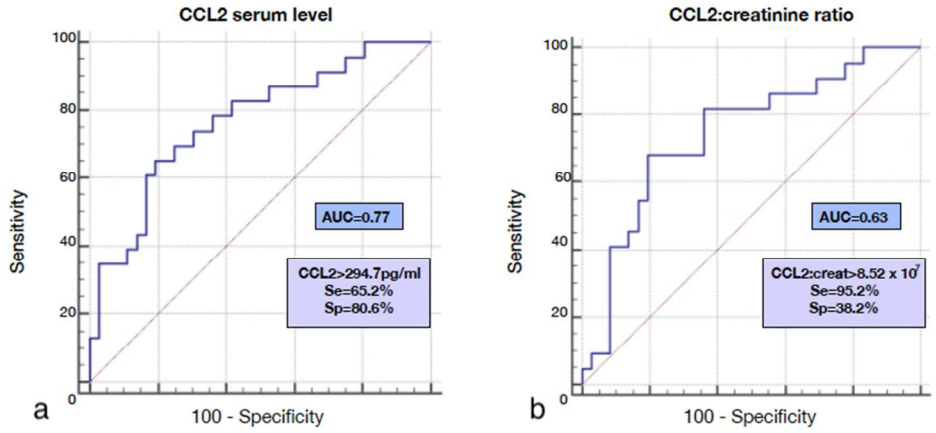


Figure 3: Correlation curve and trendline between serum CCL2 concentration and urine CCL2 : creatinine ratio (r= 0.73 ; P<0.01)

186x117mm (300 x 300 DPI)



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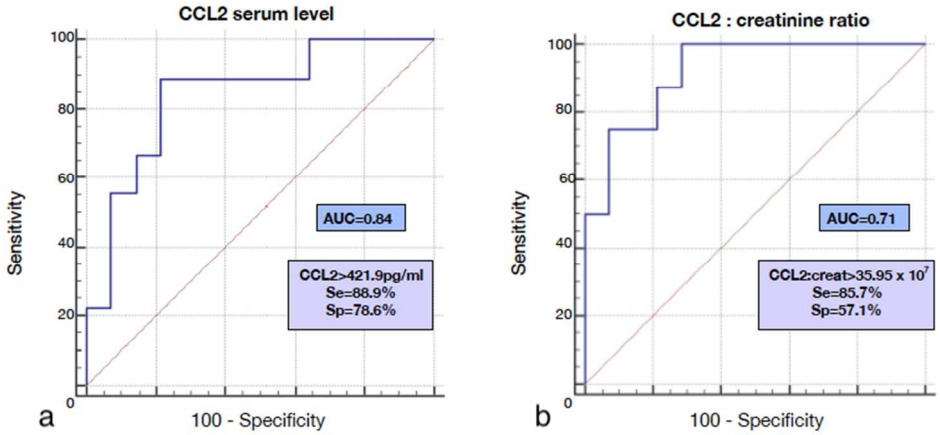

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