

# Concentration-Dependent Effect On Adherence Of Escherichia Coli To Bladder Epithelial Cells Of Cysticlean Capsules (240 Mg/Capsule Of Proanthocyanidins)

E Risco Rodríguez, L A Álvarez-Sala Walther, V C Ramos, I Bonet

## Citation

E Risco Rodríguez, L A Álvarez-Sala Walther, V C Ramos, I Bonet. *Concentration-Dependent Effect On Adherence Of Escherichia Coli To Bladder Epithelial Cells Of Cysticlean Capsules (240 Mg/Capsule Of Proanthocyanidins)*. The Internet Journal of Microbiology. 2015 Volume 13 Number 1.

DOI: [10.5580/IJMB.33094](https://doi.org/10.5580/IJMB.33094)

## Abstract

### Background

The proanthocyanidins (PACs) are the main components of cranberry (fruit of *Vaccinium macrocarpon* Aiton), and their content determines the effectiveness in the urinary tract infections (UTIs). The activity of PACs is related to inhibition of bacterial adherence. Cysticlean® is a cranberry extract product with a high quantity of proanthocyanidins (240 mg/capsule of PACs). Cysticlean® is used in the prevention and treatment of recurrent UTIs. The aim of this study is the comparison of different concentrations of Cysticlean® capsules on adherence of *Escherichia coli* to bladder epithelial cells.

### Methods

In vitro, *E. coli* pre-incubated in different concentrations of Cysticlean® capsules was incubated with human urinary bladder carcinoma cells (T24 cells) for 1 hour and the number of bacteria adhered to cells was recorded.

### Results

Cysticlean® has demonstrated a dose-dependent inhibition of bacterial adherence. At concentrations of 5, 25, 75 and 137 mg PACs/mL, Cysticlean® decreases the number of bacteria adhered to epithelial cells by 17%, 52%, 76 and 89%, respectively.

### Conclusions

Cysticlean® capsules (240 mg/capsule of PACs) induces a dose-dependent significant decrease in the number of *E. coli* adhered. Previous clinical assays showed that Cysticlean® is a product highly recommended in the prophylaxis and treatment of UTIs. The present study confirms that the amount of proanthocyanidins is very important to achieve greatest effectiveness of the cranberry products.

## BACKGROUND

UTIs (Urinary tract infections) are a frequent problem in primary care and it refers to microbial colonization of the urine and infection of the structures of the urinary tract. Adhesion of bacteria to the uroepithelial cells is a key point in the development of urinary tract infection (UTI), and it is a major virulence factor in their complex pathogenesis. Therefore one of the most obvious ways to prevent UTIs is to reduce the adhesiveness of bacterial cells that could cause the infection. Moreover recurrent UTIs are caused by

biofilm forming bacteria and there are a significant correlation between bacteria motility and biofilm production.

In a urinary tract infection (UTI), the bacterial adhesion is a critical first step prior to invasion. UTIs are mainly attributed to a highly heterogeneous group of *Escherichia coli*. Urothelial cell invasion and biofilm formation are two involved mechanisms. Bacterial motility is also associated with virulence of bacterial pathogens. The initiation of this process involves the adhesion of bacteria to the epithelium, allowing them to remain at the urinary tract despite the drag

effect of urinary flow. Those responsible for this adhesion are filamentous appendages (fimbriae or pili) on the surface of the bacteria, although there are also adherent structures, or adhesins, not fimbriated. The antiadherence activity is one of the therapeutic mechanisms to address the treatment and prophylaxis of UTIs. It is known that cranberry (fruit of *Vaccinium macrocarpon* Aiton) reduces bacteria adhesion, motility by change on bacterial cell morphology and the biomass of a biofilm.

Proanthocyanidins (PACs) are identified as the main responsible for the anti-adherence activity of cranberry. Cranberry PACs have the two types of linkages between epicatechin units, the B-type and the less common A-type. A-type linkages are particularly present in cranberry PACs and may be important structural features in the anti-adhesion process. This action could be related to the ability of proanthocyanidins (PACs) to bind to proteins, such as the adhesins present on these *E. coli* fimbriae [1-4]. A-type PACs from cranberry differs from B-type PACs found in most berry fruits, and are considered the main active ingredient for inhibiting P-fimbriated *E. coli* adherence to uroepithelial cells. They have demonstrated anti-adhesion effect of these A-type PACs against *Escherichia coli*, whereas B-type PACs from other fruits were devoid of anti-adherence properties. Moreover, the content of the PACs determines the effectiveness of a cranberry product in a concentration-dependent manner.

Cranberry products show a significant inhibition of *E. coli* adherence, in vitro and ex vivo, to uroepithelial cells and other different substrates [5-11].

Cranberry products, with the appropriate amount of PACs, have been clinically proven to be highly effective, as a co-adjuvant treatment, in helping to reduce UTIs recurrence. This use is supported by Cochrane revision (2012), ESCOP monograph, meta-analysis and different published reviews. But it is important to note that the level of the PACs determines the effectiveness of a cranberry product. Clinical evidence supports that the consumption of the PACs of the cranberry products prevents the recurrent UTIs [12-16]. This effect has mainly been studied in women, particularly in women with recurrences, but has also shown a significant reduction in the frequency of these infections in men and in children. Cranberry treatment is a safe, well-tolerated supplement that does not have significant drug interactions [17].

The first review of the Cochrane Library (2001) about cranberry for the prevention and treatment of UTIs concluded that there was preliminary evidence supporting its efficacy. A second Cochrane review of 2008 identified 10 studies on the use of cranberries to prevent UTI and highlighted that cranberries are effective for the prevention of recurrent UTI, especially in young sexually active women [18]. The last Cochrane revision highlights the need for quantified preparations using standardized methods to ensure the potency and enough content of PACs, since, probably the cause of the lack of efficacy observed in some preparations of cranberry juice is lower PACs amount administered [13, 19]. Moreover, meta-analysis was performed using the data from four randomized controlled trials [20-22], where women, subjects with spinal cord injury or the elderly people were involved [18, 23]. And the results show that cranberry products significantly reduced the incidence of symptomatic UTIs in 12 months compared with placebo or control, particularly in women with recurrent UTIs. The Society of Urologic Nurses and Associates has concluded that cranberry products have been posited to prevent or treat UTIs [16].

In preclinical studies, human uroepithelial cells and human urinary bladder carcinoma cell line (T24) are often used in experimental in vitro assays. The use of a bladder epithelial cell line increases the reproducibility of the adherence test by comparison with uroepithelial desquamated cells coming from volunteers or patients [24]. Other studies test for the ability to agglutinate red blood cells using a mannose-resistant human red blood cell (HRBC) assay. Martino et al. [24] developed a bioassay to test the adhesion to the T24 bladder epithelial cell line of bacteria grown in urine samples collected after placebo or cranberry preparation drinking in a double-blind procedure.

The cranberry extract product Cysticlean® is used in the prevention and treatment of recurrent UTIs. Cysticlean® tablets and sachets showed a significant inhibition of *E. coli* adherence, in vitro and ex vivo, to uroepithelial cells. Cysticlean® exhibited in vitro a potent inhibition of adherence of *E. coli* adherence, pre-incubated in different concentrations of product after the incubation with T24 cells for 1 hour. For these in vitro adherence assays has been used an adaptation of the method proposed by Gupta et al. [25], with T24 cells and using the *E. coli* strain ATCC 10536. Statistically significant differences were observed between the control group and all the treated groups with different

concentrations of Cysticlean® (5, 25 and 75 mg/mL of PACs) [9].

Additionally, Cysticlean® has shown a beneficial effect as a prophylactic treatment of UTIs in women and in children. Cysticlean® products produces a reduction in habitual recidivism, including recurrent post-coital UTIs, and it is also observed a great improvement in symptoms such as urinary frequency, dysuria, burning during urination, haematuria and leukocyturia in sediment. Moreover, the use of Cysticlean® is safe and well-tolerated [26-30].

The new formulation of Cysticlean as capsules provides 240 mg of PACs (vanillin-HCl modified method). The objective of this study is the comparison of the in vitro effect of different concentrations of Cysticlean capsules on adherence of Escherichia coli to bladder epithelial cells.

## METHODS

### Cysticlean® samples

The batches 140614 of Cysticlean® capsules (240 mg/capsule of PACs) and 20110428 of the previous Cysticlean® capsules (118 mg/capsule of PACs) have been used.

The test concentrations of PACs have been adjusted in McCoy's 5a modified medium, according to the human doses: 137 mg/mL (for Cysticlean 240 mg/capsule of PACs) and 75, 25 and 5 mg/mL (for Cysticlean 118 mg/capsule of PACs).

### Preparation of the cell culture

Human urinary bladder carcinoma cells (T24, ATCC HTB-4TM) ( $1.1 \times 10^6$  cells/mL) were grown in McCoy's 5a modified medium, supplemented with fetal calf serum (10%), L-glutamine (10 M), penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were grown on chambered glass slides (Chamber slide system, Lab Tek®) to obtain confluent monolayers, and were incubated at 37°C, for 72 hours in a humidified atmosphere of 5% CO<sub>2</sub>.

### Preparation of bacterial suspensions and pre-incubation period

E. coli strain ATCC 10536 was grown on TSA plates, at 37°C. Afterwards, it was collected in PBS. After washing, it was collected in McCoy's 5a modified medium and re-suspended at a concentration of approximately  $4 \times 10^8$  cfu/mL. This bacterial suspension was re-suspended in

different concentrations (volume: 1 mL) for each treatment (in triplicate form). Pre-incubation under agitation at 37°C for 30 minutes was performed.

### Test procedure

To perform the adherence assay, monolayers were washed three times with PBS. Afterwards, the pre-incubated bacterial suspension was added and incubated at 37°C for 1 hour. Cells treated with McCoy's 5a modified medium alone served as control. After incubation, the supernatants were decanted and the monolayers washed five times with PBS. The cells were fixed with methanol and stained with Giemsa 20% (v/v).

The number of bacteria adhered to the cells was recorded for 30 cells (10 cells for each replicate). The average number of bacteria adhered per cell was calculated for each treatment group. The percentage of inhibition with respect to the control group was also calculated for each concentration of the products tested. The results obtained were compared using a one-way analysis of the variance (ANOVA). Were significant differences were found, the Student-Newman-Keuls test was done  $p < 0.05$ .

## RESULTS

### In vitro inhibition of the E. coli adherence

The Table 1 shows the mean of the number of bacteria adhered to cells for each concentration of Cysticlean® capsules and the inhibition percentage. The highest number of bacteria adhered per cell was observed in the control group.

**Table 1**

Effect of Cysticlean® on the adherence of Escherichia coli Results of the in vitro assay used for the evaluation of the effect of Cysticlean® capsules on the adherence of E. coli to T24 uroepithelial cells.

Treatment	Dose (µg PACs/mL)	Mean of the number of bacteria adhered per cell (± SD)	Inhibition (%)
Cysticlean® capsules	5	$26.8 \pm 0.58$	17.3
	25	$15.5 \pm 0.44$	52.2
	75	$7.8 \pm 0.28$	75.9
	137	$6.4 \pm 1.13$	88.6

No bacteria were observed in cells only treated with McCoy's 5a Modified Medium.

Statistically significant differences were observed between the Control Group and groups treated with Cysticlean® at 5,

25, 75 and 137 µg PAC/ml (Student-Newman-Keuls test  $p < 0.01$ ).

This anti-adherence activity of Cysticlean® is a dose-dependent effect. The highest inhibitory effect was observed at a concentration of 137 mg/mL of PACs (89%). At the lowest concentration (5 mg/mL of PACs) the inhibition was 17%. Moreover, at the concentration of 25 mg/mL and 75 mg/mL of PACs) the inhibition was 52% and 75%, respectively.

## DISCUSSION

One important property of *E. coli* is its adherence to the host tissue. The bacterial adhesion is a critical first step prior to invasion. In fact, if *E. coli* cannot attach to the inner urinary wall it cannot colonize and grow. Actually, without adhesion, the bacteria cannot infect the mucosal surface.

Therefore, anti-adhesion is a functional concept for prevention of pathogens, and cranberry has demonstrated its ability to inhibit the bacterial adhesion and colonization. For this objective, robust in vitro assays for bacterial adhesion on host cells have long been used for the screening of potential therapeutic agents for the ability to minimize pathogen colonization of human tissues. Human uroepithelial cells and human urinary bladder carcinoma cell line (T24) are often used in these experimental in vitro assays.

Previous in vitro, showed that Cysticlean® tablets and sachets produces a potent dose-dependent inhibition of *E. coli* adherence, pre-incubated in different concentrations of product after the incubation with T24 cells for 1 hour. For these in vitro adherence assays has been used an adaptation [9] of the method proposed by Gupta et al. [25], with T24 cells and using the *E. coli* strain ATCC 10536.

In the present study, with the same experimental assay, Cysticlean® capsules, at concentrations of 5, 25, 75 and 137 mg/mL of PACs, decrease the number of bacteria adhered to uroepithelial cells by 17%, 52%, 76% and 89% respectively. It can be observed that the highest inhibition of Cysticlean® capsules was obtained at the concentration of 137 mg/mL of PACs.

Moreover, Cysticlean® have also demonstrated, ex vivo, a dose-effect inhibition of bacterial adherence [9].

The dose-dependent effect of PACs has already been described in several studies, although a range of lower concentrations [24, 31]. In addition, although some

laboratories proposed dose of 36 mg/day of PACs, it is interesting that subsequent studies have shown that higher doses of PACs provide greater activity, and have confirmed that the consumption of cranberry dosages containing 72 mg of PACs is more effective than 18 or 36 mg of PACs [32]. However, as already discussed and demonstrated, higher doses as provided by Cysticlean® produce a greater benefit in clinical studies and it can be established the dose of 240 mg as adequate daily amount of PACs.

The mechanism of action implicated in the effect of cranberry of *E. coli* adherence it has been reported by the interactions between cranberry and the surfaces of *E. coli*. It is probably related with the decrease in the adhesion forces of the fimbriae between the cell surface and the bacteria [6, 33-35].

Additionally, Cysticlean® has shown a beneficial effect as a prophylactic treatment of UTIs in women [26-27, 29] and in children [28]. These results showed the high efficacy and good tolerance of Cysticlean® in children and women with frequent urinary tract infections. And as has been demonstrated for other cranberry products, the use of Cysticlean® could reduce the antibiotic use. It is also important to know that the anti-adherence activity associated with cranberry consumption is not related to antibiotic sensitivity or resistance [36].

## CONCLUSIONS

Previous clinical assays showed that Cysticlean® is a product highly recommended in the prophylaxis and treatment of UTIs. For people with recurrent uncomplicated UTIs, PACs of cranberry products as Cysticlean® may offer an alternative methodology to antibiotic prophylaxis and an improvement of their symptomatology.

Cysticlean® capsules has been shown a dose-dependent effect on adherence of *Escherichia coli* to uroepithelial cells, and this activity is related to the PACs content in the cranberry extract.

The present study confirms that the amount of PACs is very important to achieve greatest effectiveness of the cranberry products in the management of UTIs.

Competing interests

The authors declare that they have no competing interests. The study was funded by Vita Green Europa.

## Acknowledgements

This study was funded by Vita Green Europa. We thank Máximo Encabo and Irène Lenthéric, from Harlan, as study director of the experimental assays.

## References

- [1] Foo LY, Lu Y, Howell AB, Vorsa N. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro. *Phytochemistry*. 2000; 54 (2): 173-181.
- [2] Howell AB, Vorsa N, Marderosian AD, Foo LY. Inhibition of the adherence of P-fimbriated *Escherichia coli* to uroepithelial cell surfaces by proanthocyanidins extracts from cranberries. *N Engl J Med*. 1998; 339 (15): 1085-1086.
- [3] Howell AB, Reed JD, Krueger CG, Winterbottom R, Cunningham DG, Leahy M. A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. *Phytochemistry*. 2005; 66 (18): 2281-2291.
- [4] Howell AB. Bioactive compounds in cranberries and their role in prevention of urinary tract infections. *Mol Nutr Food Res*. 2007; 51 (6): 732-737.
- [5] Ahuja S, Kaack B, Roberts J. Loss of fimbrial adhesion with the addition of *Vaccinium macrocarpon* to the growth medium of P-fimbriated *Escherichia coli*. *J Urol*. 1998; 159: 559-562.
- [6] Johnson BJ, Lin B, Dinderman MA, Rubin RA, Malanoski AP. Impact of cranberry on *Escherichia coli* cellular surface characteristics. *Biochem Biophys Res Commun*. 2008; 377: 992-994.
- [7] Johnson BJ, Lin B, Rubin RA, Malanoski AP. Media acidification by *Escherichia coli* in the presence of cranberry juice. *BMC Res Notes*. 2009; 2: 226.
- [8] Pinzón-Arango PA, Liu Y, Camesano TA. Role of cranberry on bacterial adhesion forces and implications for *Escherichia coli*. *Uroepithelial cell attachment*. *J. Med Food*. 2009; 12 (2): 259-270.
- [9] Risco E, Miguélez C, Sánchez de Badajoz C, Rouseaud A. Effect of American cranberry (*Cysticlean*) on *Escherichia coli* adherence to bladder epithelial cells. In vitro and in vivo study. *Arch Esp Urol*. 2010; 63 (6): 422-430.
- [10] Mathison BD, Kimble LL, Kaspar KL, Khoo C, Chew BP. Development and validation of a sensitive, high-throughput bioassay for the adhesion of radiolabeled *E. coli* to uroepithelial cells in vitro. *J Nat Prod*. 2013; 76 (9): 1605-1611.
- [11] Chan M, Hidalgo G, Asadishad B, Almeida S, Muja N, Mohammadi MS, Nazhat SN, Tufenkji N. Inhibition of bacterial motility and spreading via release of cranberry derived materials from silicone substrates. *Colloids Surf B Biointerfaces*. 2013; 110: 275-280.
- [12] Raz R, Chazan B, Dan M. Cranberry juice and urinary tract infection. *Clin Infect Dis*. 2004; 38 (10): 1413-1419.
- [13] Jepson RG, Williams G, Craig JC. Cranberries for preventing urinary tract infections. *Cochrane Database Syst Rev*. 2012; (10): CD001321.
- [14] Nowack R, Schmitt W. Cranberry juice for prophylaxis or urinary tract infections. Conclusions from clinical experience and research. *Phytomedicine*. 2008; 15: 653-667.
- [15] ESCOP (European Scientific Cooperative on Phytotherapy). *ESCOP Monographs: The scientific foundation for herbal medicinal products. Cranberry (Vaccinii macrocarpi fructus)*. 2<sup>a</sup> Edition. Supplement 2009. New York: ESCOP (Exeter), Georg Thieme Verlag (Stuttgart), Thieme New York, 2009: 255-269.
- [16] Wang P. The effectiveness of cranberry products to reduce urinary tract infections in females: a literature review. *Urol Nurs*. 2013; 33 (1): 38-45.
- [17] Pérez-López F, Haya J, Chedraui P. *Vaccinium macrocarpon*: an interesting option for women with recurrent urinary tract infections and other health benefits. *J Obstet Gynaecol*. 2009; 35 (4): 630-6309.
- [18] Jepson RG, Craig JC. Cranberries for preventing urinary tract infections. *Cochrane Database Syst Rev*. 2008; (1): CD001321.
- [19] Jepson RG, Williams G, Craig JC. Cranberries for preventing urinary tract infections. *Sao Paulo Med J*. 2013; 131 (5): 363.
- [20] Stothers L. A randomized trial to evaluate effectiveness and cost effectiveness of naturopathic cranberry products as prophylaxis against urinary tract infection in women. *Can J Urol*. 2002; 9 (3): 1558-1562.
- [21] Kontiokari T, Sundqvist K, Nuutinen M, Pokka T, Kostela M, Uhari M. Randomised trial of cranberry-lingonberry juice and *Lactobacillus GG* drink for the prevention of urinary tract infections in women. *Brit Med J*. 2001; 322: 1571-1573.
- [22] McMurdo MET, Bissett LY, Price RJG, Phillips G, Crombie IK. Does ingestion of cranberry juice reduce symptomatic urinary tract infections in older people in hospital? A double-blind, placebo-controlled trial. *Age and Ageing*. 2005; 34 (3): 256-261.
- [23] Jepson RG, Craig JC. A systematic review of the evidence for cranberries and blueberries in URI prevention. *Mol Nutr Food Res*. 2007; 51 (6): 738-745.
- [24] Di Martino P, Agniel R, David K, Templer C, Gaillard JL, Denys P, et al.. Reduction of *Escherichia coli* adherence to uroepithelial bladder cells after consumption of cranberry juice: a double-blind randomized placebo-controlled cross-over trial. *World J Urol*. 2006; 24 (1): 21-27.
- [25] Gupta K, Chou MY, Howell A, Wobbe C, Grady R, Stapleton AE. Cranberry products inhibit adherence of P-fimbriated *Escherichia coli* to primary cultured bladder and vaginal epithelial cells. *J Urol*. 2007; 177 (6): 2357-2360.
- [26] Bonet I, Batista E, Conejero J, Cortadellas L, Mandaña A, Peyrí E, Pigrau A, Urmeneta JM, Vargas C, Viladoms JM. Arándano rojo en el tratamiento de la cistitis urinaria.

Urol Integr Invest. 2008; 13 (3): 214-217.

[27] Collado A, Trassierra M, Monllor E, Navalón P, Tramoyeres A, Ordoño F, Osca J, Gómez A, Monzonís L, Dumont R. Estudio observacional del tratamiento con un extracto de arándano rojo Americano rico en proantocianidinas (PAC 118 mg) para el tratamiento de las infecciones urinarias de repetición. Urol Integr Invest. 2009; 14 (4): 366-369.

[28] Garat Barredo JM. Tratamiento de las infecciones urinarias en pediatría con extracto de arándano rojo Americano. Acta Pediatr Esp. 2011; 63 (3): 117-120.

[29] Ballester FS, Vidal VR, López Alcina E, Domenech Pérez C, Escudero Fontano E, Oltra Benavent AM, Montoliu García A, Sobrón Bustamante MA. Cysticlean a highly PAC standardized content in the prevention of recurrent urinary tract infections: an observational, prospective cohort study. BMC Urol. 2013; 13 (1): 28.

[30] Rodríguez Escovar F, de Garcia A, Villamizar JM. Cysticlean y profilaxis en infecciones urinarias recurrentes: ¿es la edad un factor limitante? Revista Médica Electrónica de Portales Médicos (<http://www.revista-portalesmedicos.com/revista-medica/cysticlean-y-profilaxis-infecciones-urinarias-recurrentes-edad/>). Accessed 24 March 2015.

[31] Lavigne JP, Bourg G, Combescure C, Botto H, Sotto A. In vitro and in vivo evidence of dose-dependent decrease of

uropathogenic Escherichia coli virulence after consumption of commercial Vaccinium macrocarpon (cranberry) capsules. Clin Microbiol Infect. 2008; 14: 350-355.

[32] Howell AB, Botto H, Combescure C, Bland-Potard AB, Gausa L, Matsumoto T, Tenke P, Sotto A, Lavigne JP. Dosage effect on uropathogenic Escherichia coli anti-adhesion activity in urine following consumption of cranberry powder standardized for proanthocyanidins content: a multicentric randomized double blind study. BMC Infect Dis. 2010; 10: 94.

[33] Liu Y, Black MA, Caron L, Camesano TA. Role of cranberry juice on molecular scale surface characteristics and adhesion behavior of Escherichia coli. Biotechnol Bioeng. 2006; 93 (2): 297-305.

[34] Liu Y, Gallardo-Moreno AM, Pinzón-Arango OA, Reynolds Y, Rodríguez G, Camesano TA. Cranberry changes the physicochemical surface properties of E. coli and adhesion with uroepithelial cells. Colloids Surf B Biointerfaces. 2008; 65 (1): 35-42.

[35] Liu Y, Pinzón-Arango OA, Gallardo-Moreno AM, Camesano TA. Direct adhesion force measurements between E. coli and human uroepithelial cells in cranberry juice cocktail. Mol Nutr Food Fes. 2010; 54 (12): 1744-1752.

[36] Hisano M, Bruschini H, Nicodemo AC, Srougi M. Cranberries and lower urinary tract infection prevention. Clinics. 2012; 67 (6): 661-667.

**Author Information**

**Ester Risco Rodríguez**

Phytonexus, S.L. Na Jordana

València, Spain

ester.risco@phytonexus.com

**Luis A Álvarez-Sala Walther**

Universidad Complutense de Madrid, Plaza de Ramón y Cajal Ciudad Universitaria

Madrid, Spain

lalvarezsalaw@gmail.com

**Vitoria Cachafeiro Ramos**

Universidad Complutense de Madrid, Plaza de Ramón y Cajal Ciudad Universitaria

Madrid, Spain

vcara@med.ucm.es

**Isidre Bonet**

Teknon Clinic, Urology Department

Barcelona, Spain

isidrebonet@hotmail.com