

Pentosan polysulfate decreases prostate smooth muscle proliferation and extracellular matrix turnover

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Benign prostatic hyperplasia (BPH) involves proliferation of smooth muscle cells and increased deposition of extracellular matrix (ECM). We recently found that pentosan polysulfate (PPS) has marked effects on growth and ECM of smooth muscle cells derived from vascular tissues. We examined smooth muscle cells cultured from human prostates and the effects of PPS on their growth and ECM production. Fragments of surgical prostatectomy specimens were diced, digested with collagenase (0.01%), and placed in culture medium supplemented with 20% fetal bovine serum. Outgrowths of elongated cells were characterized by light microscopic examination and immunohistochemical techniques by the presence of F-actin, α -smooth muscle actin, and myosin, which is a characteristic of smooth muscle cells. Two independent isolates were propagated, and growth curves and ECM production were assessed in the presence and absence of PPS (10 or 100 μ g/ml). PPS decreased cell number beginning at day 1 and throughout the incubation period, up to 4 days. The amount of the ECM degradative enzymes, metalloproteinases MMP-9 and MMP-2, was examined by zymography. PPS did not alter the amount of MMP-2 in the supernatants but MMP-9 was increased 234.4 ± 17.23 -fold over control cells. Tissue inhibitor of MMP (TIMPS), examined by reverse zymography, increased 200% over control. The amount of α I type (IV) and α I type (I) collagen released in the supernatant, measured by ELISA, significantly decreased in PPS-treated cultures. In conclusion, we found that the administration of PPS decreased proliferation as well as ECM production in prostate smooth muscle. Since smooth muscle proliferation and ECM are involved in the pathophysiology of BPH, PPS may have therapeutic potential.

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Introduction

We found that pentosan polysulfate (PPS) inhibits the proliferation of smooth muscle and matrix production in

a dose-dependent fashion.¹ With the interest in agents to reduce the size of the prostate, namely 5-alpha-reductase inhibitors and more recently phytotherapeutic agents, we decided to study the effect of PPS on human prostate tissue.

While androgen dependence on prostate growth is well documented, there has been recent interest in the regulation of growth at the cellular level. Prostate growth is modulated by endocrine factors, neuroendocrine signals, paracrine factors, autocrine factors, intracrine

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factors, extracellular matrix (ECM) factors, and cell–cell interactions.² Several studies have shown that heparin or related compounds modulate the synthesis of ECM by vascular smooth muscle cells *in vitro*, by decreasing the synthesis of types I and IV collagen.^{2–4} We previously described the effect of PPS on glomerulosclerosis, arteriosclerosis, and vascular graft stenosis.^{2,3} PPS mainly affects ECM regulation but has also been shown to have other effects including inhibition of complement activation, binding of growth factors, and inhibition of DNase activity.^{5–7}

PPS has been studied for 30 years and has only recently been approved for the treatment of interstitial cystitis (IC). While the efficacy of PPS in reducing the symptoms of IC has been attributed to its local anti-adherence effects and restoration of the glycosaminoglycan layer (GAG) of the bladder lining, other effects of PPS may be equally important. PPS has the advantage of ‘being practically nontoxic’ and serious adverse effects on patients treated for IC were mainly gastrointestinal or alopecia in 3.2% of chronic users.⁸

Thus, PPS, a drug with a low side effect profile and the potential to inhibit prostate growth, may have a role in the treatment of benign prostatic hyperplasia (BPH).

Materials and methods

Cell culture and characterization

Fragments of two prostatectomy specimens were diced, and incubated in a collagenase solution (3 mg/ml) (Sigma, St Louis, MO, USA) overnight at 37°C. The following day, the digestate was resuspended in complete Waymouths medium (Gibco) containing 20% fetal bovine serum (FBS), l-glutamine, penicillin (100 U/ml) (Inv Hrogen Corp, Cartshad, CA, USA), streptomycin (100 µg/ml), and sodium bicarbonate, and plated into 24-well plates (Nunc Nalge International, Naperville, IL, USA) coated with human fibronectin (Collaborative Research). The fragments were left undisturbed in 95% O₂, 5% CO₂ until the first outgrowth was apparent. The medium was changed twice a week until the cells reached confluence, at which time the cells were transferred into flasks. All experiments were performed on cells between passages 2 and 10.

The cells were characterized by staining for α -smooth muscle cell actin (Sigma, St Louis, MO, USA), F actin (Molecular Probes, OR, USA), and myosin (Biomedical Technologies, MA, USA) performed according to the manufacturer’s directions.

Growth curves

Dose–response curves were performed. Cells were plated at 5000 cells/well in 24-well plates (Nunc) in medium containing 20% FBS. After 36 h the medium was replaced with fresh 20% FBS-containing medium and increasing concentrations of PPS (Elmiron[®], Ivax Corp, Miami, FL, USA 10 or 100 µg/ml) were added. Cells were counted on days 1, 3, and 4 with a Coulter Z1 cell counter (Coulter, Hialeah, FL, USA).

Metalloproteinase and TIMP production

Prostate smooth muscle cells (SMCs) were plated in 6-well plates in medium containing 20% FBS and left undisturbed for 24 h days prior to the start of the experiments. On day 0, fresh medium with and without PPS (100 µg/µl) was added. PPS was added daily for 5 days. On day 4, the medium was changed to low serum (0.1% FBS), since higher serum concentrations interfere with the MMP assays. At the time of collection, the supernatant was collected and centrifuged to remove cell debris. The cell layers were used to determine cell number.

Collagen analysis

Prostate SMCs were plated at the same time as those for zymography and treated as described above. The supernatant was centrifuged to remove cell debris and transferred to a tube containing protease inhibitors (PMSF, NEM, EDTA) and frozen prior to assay.^{1,2}

Zymography for metalloproteinases

Gelatinases (MMP-9 and MMP-2) in the medium were assayed using 10% zymogram gels (Novex, San Diego, CA, USA) as previously described.¹ Briefly, the medium was diluted with 0.1% FBS and 5X Laemmli buffer so that all samples corresponded to an identical cell number. Following electrophoresis, the gels were washed for 1 h in 2.5% SDS and incubated overnight in collagenase as previously described. The gels were stained with Coomassie blue and air-dried. To check for nonspecific bands, gels were incubated in 50 mM Tris buffer, with the addition of 25 mM EDTA. Quantitative densitometry was performed using NIH image 1.6.

Reverse zymography

TIMPS in the supernatant were assessed by reverse zymography, as described.¹ Briefly, gels containing gelatinase A were prepared. The medium was diluted to normalize for cell number as described for zymography. Gels were washed for 1 h in 2.5% SDS following electrophoresis and incubated overnight at 37°C. Coomassie blue staining and air drying was as described for zymography. To quantitate the TIMP activity, densitometry was performed using NIH image 1.6.

Collagen ELISA

Type I collagen: 100 µl of medium or human collagen type I standard was incubated for 24 h in a 96-well plate (Nunc-Immuno Maxisorp, Nalge Nunc International, Naperville, IL, USA). After three washes with 0.05% Tween/PBS, the wells were blocked with 1% BSA/PBS for an additional 24 h. Following four washes with 0.05% Tween/PBS, 50 µl of human collagen type I antibody (1:1000) (Biodesign, Kennebunk, Maine, Germany) was applied for 90 min. The plate was washed again and

biotin-labeled goat anti-rabbit IgG (1:1000, Sigma) was applied for 1 h. The wells were washed four more times and incubated with streptavidin horseradish peroxidase (SAAP) at a 1:2500 concentration. *p*-nitro-phenyl phosphate (pNPP, Sigma, St Louis, MO, USA) was used for color development according to the manufacturer's directions. The plate was read in a Titertek Multiskan plate reader at 405 nm.

Type IV collagen: The assay was performed in a similar way, except that the medium and standards were coated on the plate for 8 h prior to blocking. The concentration of the antibody and the biotinylated goat anti-rabbit IgG was 1:2000. The SAAP was used at 1:1500.

Type I and type IV (Bioscience Resource Project) collagen standards were diluted to a final concentration of 0.25–4 ng/well in 0.02 N acetic acid. The curves were linear ($r = 0.98$).¹

Statistics

For both zymography and collagen analysis, each isolate was analysed in two separate experiments and each data point represents triplicate or quadruplicate (separate) wells. Differences between PPS treatment and control cells were assessed using Student's *t*-test (Prism, Graph-Pad, San Diego, CA, USA).

Results

Cell morphology

Two independent isolates of cells were propagated. Outgrowth was observed in both isolates by day 7. The cells appeared elongated, stellate, and formed parallel arrays. There were no morphologic changes in the cells during the 10 passages over which the cells were studied. The cells grew as a monolayer. At confluence they did not form hillocks.

The cells contained end-to-end actin filaments when stained with phalloidin, a characteristic of mesenchymal cells. Furthermore, since they contained smooth muscle actin (Figure 1) and myosin they were considered to be SMCs.

Cell turnover

The addition of FBS stimulated cell proliferation. There was no change in the growth rate as a function of passage number for the duration of these studies, which included primary passages. Cell proliferation was markedly inhibited at confluence. Cell number decreased 24 h following treatment with PPS (10 or 100 µg/ml, Figure 2). This was not associated with cell death, as assessed by trypan blue staining. By day 4, a three-fold decrease in cell number was noted with 100 µg/ml, and therefore this dose was chosen for all further experiments.

Zymography

MMP-9 activity increased $234.4 \pm 17.23\%$ over control ($P < 0.001$, Figure 3). However, there were no significant

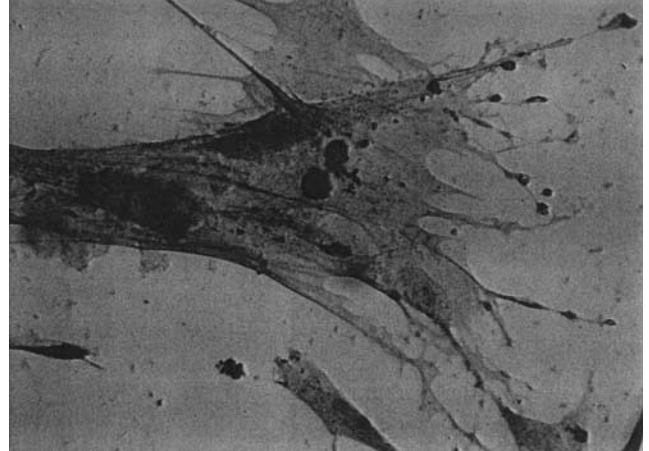


Figure 1 Prostate cells: α -smooth muscle cell actin.

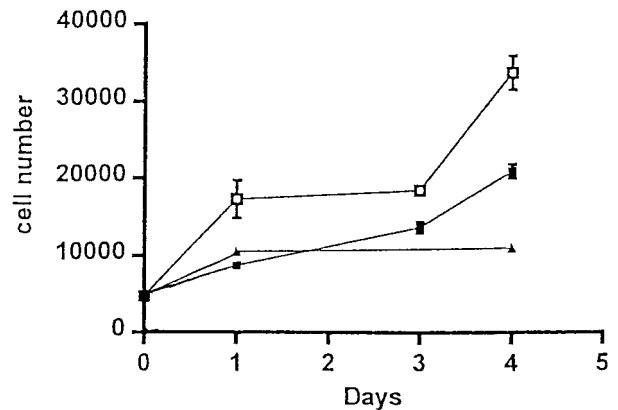


Figure 2 Representative growth curve of human prostate cells plated in 20% serum containing medium (□). Following a 24 h attachment, PPS (10 µg/ml ■; 100 µg/ml ▲) was added to the appropriate wells. Duplicate wells were counted on days 1, 3, and 4.

differences in MMP-2 activity in the medium between PPS-treated and control cells.

Reverse zymography

TIMP-1 and TIMP-2 were present in the media of both isolates examined. In prostate SMCs treated with PPS, TIMP-1 levels were two-fold higher as compared to control cells ($P < 0.005$, Figure 4). There was no change in TIMP-2 levels. In contrast, TIMP-3 was found in the medium of all cells treated with PPS and was barely detectable in control medium.

Collagen

The cells produced both α I type (IV) and α I type (I) collagen. The cells treated with PPS showed a decrease of collagen type IV in the medium (34.21 ± 4.829 , $P < 0.01$) and cell layer (42.55 ± 5.482 , $P < 0.01$), compared to untreated cells (100%) (Figure 5). Similarly, there was a decrease in type I collagen in the medium (58.20 ± 14.20 , $P < 0.05$) and cell layer (79.58 ± 9.696), compared to untreated cells (100%) (Figure 6).

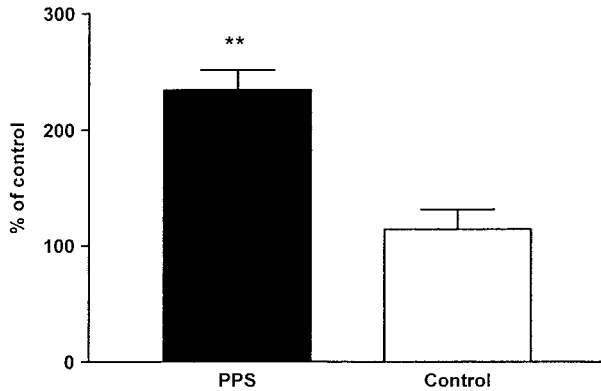


Figure 3 MMP-9 activity increased in PPS treated cells ($P < 0.001$). Data are represented as a percentage of control. Data were collected from two separate cell isolates. Each isolate was run in duplicate.

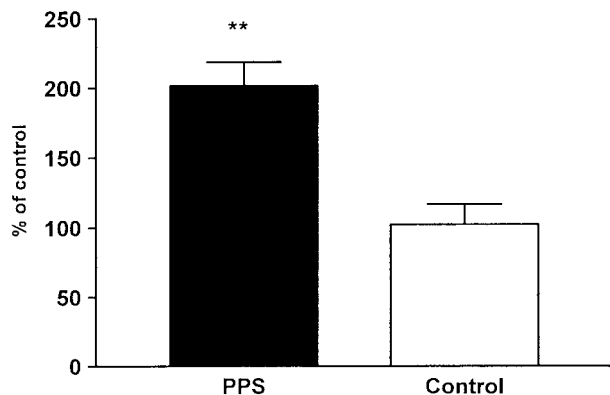


Figure 4 TIMP-1 increased in cells treated with PPS ($P < 0.005$). Data are represented as a percentage of control. Each isolate was run in duplicate.

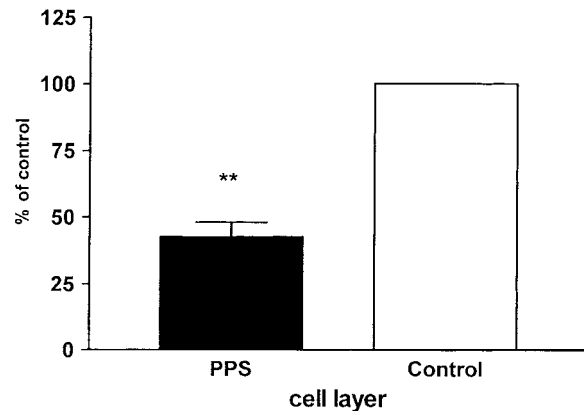
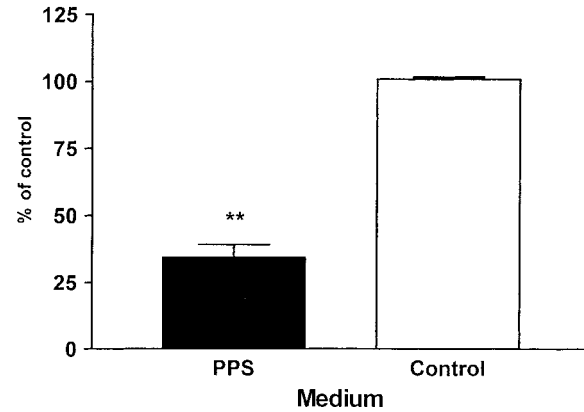


Figure 5 αI type (IV) collagen synthesis of PPS-treated isolates. PPS (black bars) induced a significant reduction of αI type (IV) collagen synthesis in the medium and cell layer when compared to untreated controls (open bars) ($P < 0.01$). Data are expressed as a percentage of control. Data were collected from two separate cell isolates. Each isolate was run twice in quadruplicate.

Discussion

The advent of medical therapy for BPH has had a major impact on the practice of urologic care. The Gallup surveys in 1994 and 1997 showed that the overwhelming majority of American urologists initiated medical therapy first in patients with moderate symptoms.⁹ One must remember that BPH is a pathologic finding and is not always found to cause benign prostatic enlargement (BPE), and that those patients with BPE may not have benign prostatic obstruction (BPO). The lack of correlation of objective parameters such as flow rate and PVR with symptoms described from data in the BTOPS study prompted Abrams and others to advocate the term lower urinary tract symptoms (LUTS)—a descriptive rather than pathologic term.¹⁰ This term serves to emphasize that BPH may be only one of numerous factors involved in patient symptomatology. Therefore, unless urodynamic testing is performed to better define bladder *vs* outlet causes of symptoms, treatment cannot be directed at a heterogeneous group of patients.

Currently available medical therapy includes alpha-blocking agents, hormonal therapy using 5-alpha-reductase inhibitors, and most recently phytotherapeutic agents. The alpha-blocking agents cause relaxation of muscle tone in the prostate stroma, prostate capsule, bladder neck, and periurethral tissues. Numerous

studies have shown that patients with and without urodynamic evidence of obstruction benefit equally with alpha-blocker treatment. This demonstrates that alpha-blockers work by other means than reduction of bladder outlet obstruction. The 5-alpha-reductase inhibitors block the conversion of testosterone to dihydrotestosterone (DHT). Moore found a 72% decrease in DHT, prostate volume reductions of 30%, and reduction of prostate-specific antigen (PSA), in patients treated with a type II 5-alpha-reductase inhibitor.¹¹ Urodynamic studies show a decrease in obstruction, but few patients will fall into the unobstructed range.¹² The symptomatic response to finasteride has been shown to be greater in prostates weighing more than 40g.¹³ However, the effect of finasteride on prostatic symptoms may be mediated by other factors than mere reduction in volume. The mechanism of action of phytoestrogens remains unclear, but at least one randomized double-blinded study reported nearly equal improvements in uroflow, symptom scores, and quality of life when compared to 5-alpha-reductase inhibitors.¹⁴ While it is often considered that alpha-blockers treat the dynamic component of BPH and 5-alpha-reductase inhibitors and possibly phyto-estrogens treat the static component, many other actions of these pharmacologic agents may be contributing to the treatment outcome.

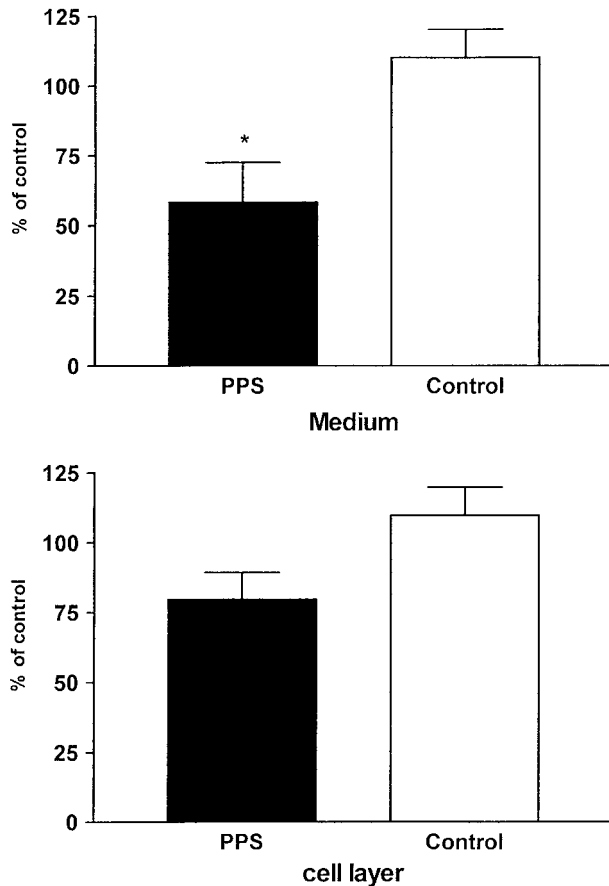


Figure 6 $\alpha 1$ type (I) collagen synthesis of cells treated with PPS (black bars). Cells treated with PPS for 5 days were collected as described and collagen measured by ELISA. PPS-treated cells showed a significant decrease of collagen in the medium ($P < 0.05$). Data are represented as a percentage of control (open bars). Data were collected from two separate cell isolates. Each isolate was run twice in quadruplicate.

PPS may also have additional actions in the relief of IC symptoms beyond the defense mechanism attributed to restoration of the GAG layer. PPS, a semi-synthetically modified polymer derived from beech tree bark, is a macromolecular carbohydrate derivative that chemically and structurally resembles glycosaminoglycans. Several studies have shown that heparin-like compounds can modulate the synthesis of ECM by vascular SMC *in vitro*, by decreasing the synthesis of types I and IV collagen. The mechanism(s) of these effects are unknown, but appear independent of anticoagulation or hemodynamic changes. Heparin may also regulate collagenase activity. This effect appears to be cell type specific, with an increase in collagenase activity in osteoblasts but a decrease in collagenase activity in vascular smooth muscle. Cultured cells from stenotic arteriovenous grafts treated with PPS showed decreased proliferation. Matrix production decreased and MMP-9 was significantly increased following treatment with PPS.²

Since the underlying pathology in BPH involves proliferation of SMC and increased deposition of ECM, the effects of PPS on these processes were evaluated. Growth curves were performed in the presence and absence of PPS (100 $\mu\text{g}/\text{ml}$ per ml) on two independent isolates. PPS decreased the cell number beginning at day 1. The degradation of ECM by 72 and 92 kDa

gelatinases (MMP-2 and MMP-9) was examined by zymography. PPS did not alter the amount of detectable MMP-2 in the supernatants but increased MMP-9 234% when compared to untreated cells. In addition, TIMP-1 was increased 200% over control and TIMP-3 was released from the ECM into the medium. The amount of type I and type IV collagen released in the supernatant was measured by ELISA. Both types of collagen were decreased in PPS-treated cultures.

Conclusion

We found that the administration of PPS decreases prostate smooth muscle proliferation as well as ECM production. Since smooth muscle proliferation and ECM are involved in the pathophysiology of BPH, PPS has the potential to produce therapeutic benefits.

References

- 1 Elliot SJ *et al*. Pentosan polysulfate decreases proliferation and net extracellular matrix production in mouse mesangial cells. *J Am Soc Nephrol* 1999; **10**: 62–68.
- 2 Elliot SJ *et al*. Pentosan polysulfate decreases proliferation and extracellular matrix deposition by vascular smooth muscle cells isolated from failed hemodialysis access grafts. *Clin Nephrol* 2000; **54**: 121–127.
- 3 Striker GE *et al*. Glomerulosclerosis, arteriosclerosis, and vascular graft stenosis; treatment with oral heparinoids. *Kidney Int* 1997; **63**: S120–S123.
- 4 Tan EML *et al*. Modulation of extracellular matrix gene expression by heparin and endothelial cell growth factor in human smooth muscle cells. *Lab Invest* 1991; **64**: 474–482.
- 5 Lush RM *et al*. A phase I study of pentosan polysulfate sodium in patients with advanced malignancies. *Ann Oncol* 1996; **7**: 939–944.
- 6 Zenjari C *et al*. Experimental evidence for FGF-1 control of blastema cell proliferation during limb regeneration of the amphibian *Pleurodeles waltl*. *Int J Dev Biol* 1996; **40**: 965–971.
- 7 Kilgore KS *et al*. The semisynthetic polysaccharide pentosan polysulfate prevents complement-mediated myocardial injury in the rabbit perfused heart. *J Pharmacol Exp Ther* 1998; **285**: 987–994.
- 8 Sand PK, Winkler HA. Analysis of long-term Elmiron therapy for interstitial cystitis. *Urology* 1997; **49**(Suppl 5A): 93–99. *J Womens Health* 1998; **7**: 268–269.
- 9 Gee WF *et al*. 1997 American Urological Association Gallup survey: changes in diagnosis and management of prostate cancer and benign prostatic hyperplasia, and other practice trends from 1994 to 1997. *J Urol* 1998; **160**: 1804–1807.
- 10 Barry MJ *et al*. Relationship of symptoms of prostatism to commonly used physiological and anatomical measures of the severity of benign prostatic hyperplasia. *J Urol* 1993; **150**: 351–358.
- 11 Moore E *et al*. Proscar: five-year experience. *Eur Urol* 1995; **28**: 304–309.
- 12 Tammela TL *et al*. Repeated pressure-flow studies in the evaluation of bladder outlet obstruction due to benign prostatic enlargement. Finasteride Urodynamics Study Group. *Neurourol Urodyn* 1999; **18**: 17–24.
- 13 Boyle P, Gould AL, Roehrborn CG. Prostate volume predicts outcome of treatment of benign prostatic hyperplasia with finasteride: meta-analysis of randomized clinical trials. *Urology* 1996; **48**: 398–405.
- 14 Carraro JC *et al*. Comparison of phytotherapy (Permixon) with finasteride in the treatment of benign prostate hyperplasia: a randomized international study of 1098 patients. *Prostate* 1996; **29**: 231–240.