# Seminal Plasma Lipocalin-Type Prostaglandin D Synthase: A Potential New Marker for the Diagnosis of Obstructive Azoospermia

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**Purpose:** We examined the relationship between L-PGDS (lipocalin-type prostaglandin D synthase) levels in seminal plasma and the presence or absence of obstruction in the male seminal tract.

**Materials and Methods:** Semen samples were collected and analyzed from 1) 10 patients with normal semen parameters, 2) 9 with obstructive azoospermia, 3) 20 after vasectomy and 4) 14 with nonobstructive azoospermia. Seminal L-PGDS was measured using an enzyme-linked immunosorbent assay technique.

**Results:** We found that seminal plasma L-PGDS in the groups with obstruction was significantly lower than in any of the other groups ($p < 0.001$). Using a cutoff of 100 $\mu$g/l all men with obstructive azoospermia had L-PGDS less than 100 $\mu$g/l, while none with normal sperm parameters did. Men with nonobstructive azoospermia had less homogeneity of L-PGDS levels, including 29.6% with L-PGDS more than 100 $\mu$g/l.

**Conclusions:** Our results suggest that seminal L-PGDS level can potentially be a biomarker for assessing patency in the seminal tract in men with azoospermia. In men with azoospermia and high seminal L-PGDS (more than 100 $\mu$g/l) the diagnosis of nonobstructive azoospermia can be potentially made without biopsy. Our study shows that using semen L-PGDS levels provides a diagnosis of nonobstructive azoospermia in almost 30% of these men.

*Key Words: testis; infertility, male; prostaglandin R2 D-isomerase; azoospermia; semen*

Infertility is a common condition, affecting up to 15% of couples.[1] Male factor is implicated in approximately 50% of the cases.[2] Azoospermia is a clinical presentation characterized by the absence of any spermatogenic elements in at least 2 semen analyses. It is diagnosed in up to 5% of men presenting for infertility investigations.[3]

The etiology of azoospermia can be classified into 2 types, including OA secondary to blockage in the sperm transport and NOA, which is due to primary testicular failure or secondary to hypothalamic-pituitary diseases. Patients with OA are usually characterized by having normal testicular volume and normal gonadotropin concentrations. However, about 29% of men with normal FSH and normal testicular size have defective spermatogenesis on testicular biopsy.[2] Therefore, the definitive diagnosis of obstructive azoospermia can only be made by performing testicular biopsy, which is an invasive procedure.

A biomarker that can be easily obtained from the patient such as seminal fluid would be a less invasive alternative to differentiate OA and NOA. We have previously studied a number of seminal plasma proteins, such as L-PGDS, prostate specific antigen, PepC, BRCA1-LIP and IGFBP-3.[4] Of them L-PGDS, which is mainly produced by the Sertoli's cells in the testis, appears to be the most promising candidate. L-PGDS is a secretory glycoprotein with a molecular mass of 26 kDa. This protein was first described under the name $\beta$-trace by Clausen as a major protein present in cerebrospinal fluid.[5] It belongs to the lipocalin superfamily, which includes an array of extracellular transport proteins showing high binding affinity for specific cell receptors and small hydrophobic ligands.[6] It is now well-known that L-PGDS is abundant in compartments beyond blood-tissue barriers, such as in the cerebrospinal fluid,[7] aqueous humor,[8] amniotic fluid[9] and seminal fluid.[10] Its concentrations in these body fluids is useful for diagnosing neurological disorders,[11] and cardiovascular,[12] renal[13] and lung[14] diseases. The role of L-PGDS in male reproduction is still unclear. Most investigators believe that the major function of L-PGDS in male fertility would be related to its capability of providing thyroid hormones and retinoids beyond the blood-testis barrier to developing germ cells in the seminiferous tubules and to maturing spermatozoa in the epididymis.[15]

Previous studies have localized L-PGDS by immunohistochemical studies to the testis and epididymis, and the epithelium of the prostate gland with the most abundant expression in the testis, specifically in Sertoli's cells.[4,16] Other studies have also demonstrated L-PDGS expression in Leydig cells.[16] L-PGDS is secreted into seminal fluid, where its concentration represents approximately 0.1% of total secreted proteins.[16] We hypothesized that L-PGDS in

Submitted for publication July 9, 2007.

Study received approval from the Mount Sinai Hospital Research and Ethics Board.

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† Financial interest and/or other relationship with Bayer Canada.



**DOI:10.1016/j.juro.2007.10.070**

semen would predominantly arise from Sertoli's cells and obstruction of the reproductive tract would decrease the seminal plasma protein concentration of L-PGDS. To investigate whether L-PGDS represents a useful biomarker we studied the relationship between its levels in seminal plasma and the presence or absence of obstruction in the male genital tract.

## MATERIALS AND METHODS

The current study was reviewed and approved by the Mount Sinai Hospital Research and Ethics Board for the development of a new test for the diagnosis of obstructive azoospermia.

### Patient Selection

Samples of seminal plasma were collected after obtaining informed consent from 53 patients. Each patient had been previously evaluated with fertility history, physical examination and hormonal profiles (FSH, testosterone and prolactin) as clinically applicable. Based on diagnosis the patients were stratified into 4 groups. 1) Group 1 consisted of 10 fertile donors with normal sperm parameters according to WHO criteria.[17] 2) Group 2 included 9 patients who were diagnosed with OA. This diagnosis was confirmed by a normal testicular biopsy or the development of azoospermia after initially successful vasovasostomy or vasoepididymostomy with a history of fertility. 3) Group 3 consisted of 20 patients who had undergone vasectomy and showed azoospermia or rare nonmotile sperm on 2 semen analyses. 4) Group 4 included 14 patients with NOA due to primary or secondary testicular failure. These diagnoses were confirmed by increased serum gonadotropins and the presence of azoospermia. All patients in this group had a normal karyotype of 46 XY except 1 with Klinefelter's syndrome (47 XXY).

### Sample Collection

In all groups semen samples were obtained by masturbation after 2 to 4 days of sexual abstinence. Ejaculates were allowed to liquefy at room temperature for 30 minutes. Seminal plasma was obtained by centrifugation at 600 $\times$ gravity at 4C for 15 minutes. It was then aliquoted and stored at $-80$C. Before assay the samples were thawed overnight at 4C and centrifuged at 7,000 $\times$ gravity for 10 minutes to separate any remaining spermatozoa from the seminal plasma.

### PGDS Assay

The concentration of PGD synthase was measured with highly sensitive and specific noncompetitive immunoassay.[9] The assay incorporated 2 PGD synthase specific monoclonal antibodies raised in mouse, including 1 for coating (clone 5F7H6) and 1 for detection (clone 7E11A12) in a sequential 2-site immunometric format with time resolved fluorescence detection. The assay has a detection limit of 0.5 $\mu$g/l and a dynamic range of up to 100 $\mu$g/l. Precision was less than 15% within the measurement range. Standards and samples were analyzed in duplicate. Seminal plasma samples were analyzed in 3 dilutions and the final concentration was determined from the linear range of the dilution.

White polystyrene microtiter plates were coated with anti-PGD synthase monoclonal antibody (5F7H6). To each well 500 ng/100 $\mu$l coating antibody solution, composed of 50 mM tris buffer and 0.05% sodium azide (pH 7.8), were added and incubated overnight at room temperature. The plates were washed 3 times with washing buffer, composed of 5 mM tris buffer, 150 mM NaCl and 0.05% Tween-20 (pH 7.8). Calibrators or samples and assay buffer (50 $\mu$l each), composed of 50 mM tris, 6% bovine serum albumin, 0.01% goat IgG, 0.005% mouse IgG, 0.1% bovine IgG, 0.5M KCl and 0.05% sodium azide (pH 7.8), were added to the wells and incubated for 2 hours with shaking at room temperature. The plates were washed 6 times with washing buffer and 100 $\mu$l biotinylated detection antibody solution, composed of 50 ng anti-PGD synthase (7E11A12) monoclonal antibody in assay buffer, were added to each well and incubated for 1 hour at room temperature with shaking. The plates were then washed 6 times with washing buffer. Subsequently 5 ng/100 $\mu$l per well of alkaline phosphatase-conjugated streptavidin solution (Jackson ImmunoResearch, West Grove, Pennsylvania) in bovine serum albumin buffer, composed of 6% bovine serum albumin, 50 mM tris and 0.05% sodium azide (pH 7.8), were added to each well and incubated for 15 minutes with shaking at room temperature. The plates were washed 6 times with wash buffer. Subsequently 100 $\mu$l substrate buffer, composed of 0.1 mol/l tris buffer (pH 9.1) containing 1 mmol/l diflunisal phosphate, 0.1 mol/l NaCl and 1 mmol/l $MgCl_2$, were added to each well and incubated for 10 minutes with shaking at room temperature. Developing solution (100 $\mu$l), composed of 1 mol/l tris base, 0.4 mol/l NaOH, 2 mmol/l $TbCl_3$ and 3 mmol/l ethylenediaminetetraacetic acid, were added to each well and incubated for 1 minute with shaking at room temperature. Fluorescence was measured with a CyberFluor™ 615 Immunoanalyzer time resolved fluorometer.

### Statistical Analysis

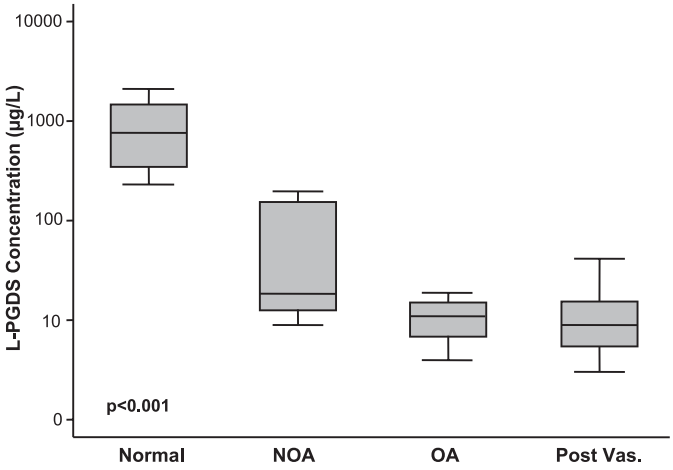
Statistical analysis was performed using SPSS® 10.0 for Windows®. Due to the nonnormal distribution of L-PGDS nonparametric statistics were used. The Kruskal-Wallis test was used to compare all 4 groups, while individual paired comparisons between groups were done using the rank sum test. The null hypothesis was rejected at $p < 0.05$.

## RESULTS

The figure shows seminal plasma L-PGDS results in $\mu$g/l in each of the 4 study groups. Overall there was a significant association between the L-PGDS level and clinical patient group ($p < 0.001$). Given this finding, to further explore the differences in the setting of OA and NOA paired comparisons were made of the 4 clinical patient groups.

### OA

The 2 groups with OA had significantly lower L-PGDS than normal control levels. Specifically median L-PGDS in the OA group was 11.0 $\mu$g/l (IQR 7–15), in the post-vasectomy group it was 9.0 $\mu$g/l (IQR 5.5–15.5) and in the normal control group it was 800 $\mu$g/l (IQR 350–1,450) (each $p < 0.001$). In addition, there was no overlap in seminal L-PGDS between the normal group and the 2 groups with evidence of obstruction in the seminal tract (OA and post-




Seminal L-PGDS in 4 study group patients plotted on logarithmic scale. Kruskal-Wallis test p value. *Post Vas.*, after vasectomy.

vasectomy groups). The lowest level of L-PGDS in the normal group was more than 20 times the highest L-PGDS value in the OA group. This gave this test 100% sensitivity for differentiating patients with obstruction from normal controls. There was no significant difference in L-PGDS between the 2 obstructive groups.

### NOA

When comparing the relatively narrow distribution of L-PGDS levels in men with OA vs that in the post-vasectomy group (r = 4 to 19 vs 3 to 42 $\mu$g/l), L-PGDS levels in men with NOA were highly variable (r = 9−200 $\mu$g/l). Median L-PGDS in men with NOA was significantly lower than in normal controls (18.5 $\mu$g/l, IQR 13−154 vs 800 $\mu$g/l, IQR 350−1,450, $p < 0.001$). Men with NOA had significantly higher L-PGDS than those in either obstructive group (OA group 11.0 $\mu$g/l, IQR 7−15, $p = 0.02$ and post-vasectomy group 9.0 $\mu$g/l, IQR 5.5−15.5, $p = 0.005$). However, using 100 $\mu$g/l as the normal reference 71.4% of the men with NOA had low L-PGDS, similar to that in the obstructive groups, while in the remaining 29.6% levels overlapped those in normal controls.

To better characterize the group with NOA we divided them into 2 subgroups, including high L-PGDS (more than 100 $\mu$g/l) and low L-PGDS (less than 100 $\mu$g/l). We compared hormonal profiles (FSH, testosterone and prolactin) and seminal plasma inhibin B with seminal L-PGDS. No significant correlation was observed.

## DISCUSSION

In our study seminal plasma L-PGDS was significantly lower in the OA and post-vasectomy groups than in normal controls. The differences were quite distinct, such that there was there no overlap between the obstructive and normal groups. This finding supports our hypothesis that most L-PGDS in semen originates from the testis with a minimal contribution from the prostate or vas deferens, making L-PGDS a potential biomarker for obstruction.

The seminal L-PGDS concentration has previously been reported to positively correlate with sperm density,[18] morphology[4] and seminal plasma $\alpha$-glucosidase,[19] which is considered an epididymal marker. These data also suggest that there is a spectrum of L-PGDS concentrations in seminal plasma in patients without azoospermia. As a result, it is difficult to interpret the decrease in L-PGDS as being secondary to obstruction or to an element of spermatogenic and Sertoli's cell dysfunction. Our current study only supports the strength of using the seminal L-PGDS level to differentiate patients with normal sperm production from those with complete obstruction.

Chen et al previously reported a significant difference in lipocalin-type PGD between their OA and NOA cases.[19] Our current study agrees with their findings. However, the levels of seminal L-PGDS in patients with NOA were widely dispersed and there was an overlap with normal controls and the obstructive group, as shown in our study. This may have been because our NOA group was a heterogeneous population. A normal L-PGDS level was observed in approximately 30% of patients with NOA, while the other 70% had lower levels (less than 100 $\mu$g/l). In patients with higher seminal L-PGDS an obstructive component can likely be ruled out based on this test alone. This can potentially eliminate the need for invasive testicular biopsy in at least 30% of men with azoospermia, making seminal L-PGDS a valuable diagnostic tool. However, in men with lower seminal L-PGDS this test alone will not be able to distinguish patients with OA from those with NOA.

Spermatogenic function is compromised in patients with NOA, resulting in the failure of germ cell maturation at various stages. The lower seminal L-PGDS level observed in 70% of patients with NOA could have been due to coexisting obstruction in the seminal tract. Alternatively these patients might have had a degree of Sertoli's cell dysfunction, resulting in lower L-PGDS production, leading to spermatogenic failure. We investigated the Sertoli's cell dysfunction hypothesis by examining the correlation between seminal L-PGDS and the levels of inhibin, which is a marker of Sertoli's cell function.[20] However, no statistically significant relationship was found. Another indirect comparison between gonadotropin and seminal L-PGDS in patients with NOA was also not contributory. Therefore, to further elucidate this question we must directly examine L-PGDS production by Sertoli's cells in the testes of patients with NOA.

Furthermore, patients with NOA with a normal testis volume and normal gonadotropins are those who present with the greatest diagnostic challenge. This group of patients was not included in our current study. We must prospectively examine this group to assess the value of seminal L-PGDS for differentiating OA and NOA.

## CONCLUSIONS

Overall our results suggest that in men with azoospermia in whom seminal L-PGDS is high or normal (more than 100 $\mu$g/l) an obstructive origin can potentially be ruled out. Thus, the seminal L-PGDS level can potentially be used as a biomarker for assessing seminal tract patency and diagnosing patients with OA. More importantly in approximately 30% of patients with NOA diagnostic testicular biopsy can be avoided based on a high or normal seminal L-PGDS level alone. Further studies are needed to delineate the role of this new biomarker in the clinical treatment of patients presenting with infertility.

**Abbreviations and Acronyms**

| | | |
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| FSH | = | follicle stimulating hormone |
| L-PGDS | = | lipocalin-type PGD synthase |
| NOA | = | nonobstructive azoospermia |
| OA | = | obstructive azoospermia |
| PGD | = | prostaglandin D |

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