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ORIGINAL ARTICLE

Testicular and epididymal dysfunctions: searching a new index for the differential diagnosis

Disfunciones testiculares y epididimarias: búsqueda de un nuevo índice para el diagnóstico diferencial

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Abstract

Objective: *This proof-of-principle aims to develop an index to aid the differential diagnosis of disorders affecting testicular and/or epididymis. A total of 202 subject data were evaluated in two groups: fertile men with children naturally conceived within 1 year of unprotected intercourse (n = 36) and infertile men (n = 166) who had attempted a pregnancy more than 1 year with unprotected intercourse.* **Materials and methods:** *Semen parameters (sperm count, vitality, motility, morphology, and hypoosmotic swelling test [HOST]) were evaluated. The index was calculated by dividing the percentage HOST by the percentage of sperm progressive motility in the fertile group (n = 36).* **Results:** *A normal range from 1.23 to 1.53 was determined. Using this index, the outcomes of semen analysis from infertile men were grouped in three study groups: below 1.23 (n = 24), normal (n = 44), and higher than 1.53 (n = 98). These parameters were significantly decreased in semen with normal range (p < 0.01) and in indexes higher than 1.53 (p < 0.0001). Receiver operating characteristic curves compared progressive motility and morphology in infertile men with indexes higher than 1.53 shows that semen samples with normal sperm progressive motility and morphology did not suggest dysfunctions in testis and epididymis. Semen samples with asthenozoospermia suggested epididymal dysfunction (area under the curve [AUC] 0.889, confidence interval [CI] 0.783-1), whereas semen samples with teratoasthenozoospermia suggested dysfunction in both testicles and epididymis (AUC 0.891, CI 0.77-1).* **Conclusions:** *The current index proof-of-principle of the success of such a strategy provides valuable information about whether a disorder individually affects testicular and/or epididymal function.*

Keywords: *Semen analysis. Progressive motility. Hypoosmotic swelling test. Male infertility. Chronic epididymitis.*

Resumen

Objetivo: *Esta prueba de principio tiene como objetivo desarrollar un índice que ayude al diagnóstico diferencial de los trastornos testiculares y/o epidídimales.* **Métodos:** *Se evaluaron 202 individuos divididos en dos grupos: hombres fértiles con hijos concebidos de forma natural en el plazo no mayor a un año (n = 36) y hombres infértiles (n = 166), los cuales habían intentado un embarazo por más de un año. Se evaluaron los parámetros seminales (concentración, viabilidad, movilidad, morfología y prueba de hinchazón hipoosmótica [HOST]). El índice se calculó dividiendo el porcentaje de HOST por el porcentaje de movilidad espermática progresiva en el grupo fértil (n = 36).* **Resultados:** *Se determinó un rango normal de 1,23 a 1,53. Utilizando este índice, los resultados del análisis del semen de los hombres infértiles se agruparon en tres grupos de estudio: por debajo de 1,23 (n = 24), normal (n = 44) y superior a 1,53 (n = 98). En contraste, estos parámetros disminuyeron significativamente en el semen de rango normal (p < 0,01) y en los índices superiores a 1,53 (p < 0,0001).*

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Las curvas ROC comparadas con la movilidad espermática progresiva y la morfología en los hombres infértiles con índices superiores a 1,53 muestran que las muestras de semen con movilidad progresiva y morfología normales no sugieren disfunciones en los testículos y epidídimos. Las muestras de semen con astenozoospermia sugerirían una disfunción del epidídimo (AUC 0,889, IC 0,783-1), mientras que las muestra de semen que presentaban teratoastenozoospermia sugerirían una disfunción tanto en los testículos como en el epidídimo (AUC 0,891, IC 0,77-1). **Conclusión:** *Estos resultados proporcionan una estrategia valiosa sobre si un trastorno afecta individualmente a la función testicular y/o epididimaria.*

Palabras clave: *Análisis de semen. Movilidad progresiva. Prueba de hinchazón hipoosmótica. Infertilidad masculina. Epididimitis crónica.*

Introduction

In the clinical investigation of male infertility semen analysis plays a key role, because it provides helpful outcomes regarding sperm production and quality. Usually, the analysis of sperm characteristics such as sperm concentration, vitality, total and progressive motility, and morphology is the main focus of the semen analysis routine[1]. Eventually, it also includes laboratory tests that assess the functional spermatozoa parameters like the hypoosmotic swelling test (HOST)[2] and DNA fragmentation test[3,4].

The analysis of total motility and also the patterns of sperm progression is an integral procedure of semen analysis[1]. They are able to assess the ability of spermatozoa to pass through the female reproductive tract until reach the site of fertilization. The sperm acquires movement and progression throughout its transit through the epididymis. Therefore, disorders affecting the functional capacity of the epididymis negatively may impact the sperm motility and male fertility[5-8], particularly in men who suffering from chronic epididymitis[9]. Thus, the assessment of sperm motility characteristics is a reliable index for assessing epididymal function, although it is typically not taken into consideration in both clinical and laboratory practices.

On the other hand, HOST assesses the ability of the sperm membrane of maintaining both physical and physiological interaction between spermatozoa and the environments on both male and female reproductive tracts. Underneath exposure to hypoosmotic conditions, the influx of fluids happens through the sperm membrane with intact integrity of viable spermatozoa to reach the osmotic equilibrium. The sperm fibers under tension cause a sperm curling (swelling), therefore distinguishing viable sperm for fertilization. Once the sperm membrane loses its integrity, the hypoosmotic solution did not cause the sperm membrane swelling (non-viable sperm), which negatively impacts the fertility status of men. Indeed, previous studies have shown that the integrity of the sperm membrane is pivotal for the events preceding fertilizations such as sperm capacitation, acrosome reaction, and binding of the sperm to the egg[2,10-12]. Therefore, HOST is a helpful index for investigating the functionality of the spermatozoa[12]. In fact, in patients undergoing assisted reproductive technologies (ART), HOST is usually performed for selecting of viable sperm for intracytoplasmic sperm injection (ICSI), to maximize the prospect of conceiving[13]. The choice of viable sperm from immotile testicular spermatozoa for ICSI leads to achieve enhanced fertilization rates, pregnancy, and ongoing pregnancy rates. Accordingly, the selection of viable based on HOST is more effective than the selection based on the sperm morphology analysis[10,14]. Conjointly, these studies show that ensuring a normal sperm membrane capacitance for fertilizing is an attribute of the testicular function.

This proof-of-principle aims to develop an index to aid the differential diagnosis for assessing testicular versus epididymal functions evaluating records of semen analysis to compare the outcomes of HOST in relation to progressive sperm motility. The usefulness of this index in determining whether dysfunctions affect either or both organs in infertile men was investigated.

Materials and methods

Subjects

A total of 202 semen analyses were included from January to December 2020. One hundred and sixty-six semen analysis records of infertile patients who had attempted a pregnancy more than 1 year with unprotected intercourse and 36 men with proven fertility (< 1 year of spontaneous conception) as a control group were included in the study. This retrospective study was approved by the Ethics Committee of the Institutional Review Board of the Lisa Andrology Lab.

Semen analysis and processing

Semen samples were obtained by masturbation, after sexual abstinence from 3 to 7 days, and processed after complete liquefaction at 37°C for sperm concentration,

vitality, and motility in accordance with the World Health Organization guidelines[15]. Azoospermic samples were excluded from the study.

Sperm morphology analysis was assessed in smears prepared from fresh semen, which was stained using a panoptic, kit (Newprov, Brazil) as reported previously[16].

The HOST was performed according to the methodology reported by Jeyendran et al.,[17]. Briefly, 0.1 mL semen sample was added to 1.0 mL of hypoosmotic solution and incubated by 30 min at 37°C. Soon after, a drop of the mixture was placed on a Neubauer chamber and the percentages of swollen sperms were assessed by counting of 200 spermatozoa.

Index calculation

Taking into account that the HOST is a marker of testicular function and the sperm motility is a marker of epididymal function, this study developed an index based on the outcomes of these semen parameters from fertile men dividing the percentage of HOST divided by the percentage of progressive sperm motility.

Based on this, infertile men were grouped according to the index into three groups: lower, normal, and higher index.

Receiver operating characteristic (ROC) curve

Finally, using ROC curves, this study also investigated whether indices greater than the reference values can assist in the differential diagnosis of disorders uniquely affecting the epididymis from disorders simultaneously affecting the epididymis plus testicles. For performing this evaluation, the study took into account to the analysis of sperm motility as an indicator of disorders affecting epididymal function and sperm morphology as an indicator of disorders affecting testicular function. Based on the results, infertile men were classified into four study groups as follows: (i) men with asthenozoospermia (n = 26); (ii) men with teratoasthenozoospermia (n = 36), and (iii) men with normal sperm motility and morphology (n = 26), and 4-men with inconclusive results (n = 10). The 95% reference intervals were calculated by removing the upper and lower 2.5% of the range to give 2.5 and 97.5 percentiles.

Statistical analysis

To determine the reference intervals of the index, this study used records of semen analysis from a population of fertile men. Data were tested for normal distribution, according to the Shapiro–Wilks method, and the confidence intervals were calculated to compose the study groups. Both one-way analysis of variance and Kruskal–Wallis test were carried out for statistical analysis of the assessed variables in the study groups for normality and non-normality distribution, respectively. If necessary, Student's t-test and Mann–Whitney U-test were used to compare variables between two study groups, for normal and non-normal distribution, respectively. $p < 0.05$ was taken as significant. Since semen with indices lower than the reference values suggests abnormal testicular function and semen with indices higher than the reference values suggests abnormal testicular and/or epididymal function this study used ROC curves to determine whether indices greater than the reference value may assist in the differential diagnosis between disorders uniquely affecting the testes or epididymis from disorders affecting both.

Results

The control group was primarily used to determine the reference interval, data were tested for normal distribution with Shapiro–Wilks method, and a reference interval from 1.23 to 1.53 was determined.

On the other hand, infertile patients were grouped according to index in three study groups: Group I (infertile patients with an index lower than 1.23 [n =2 4]); Group II (infertile patients with a normal index ranging from 1.23 to 1.53 [n = 44]), and Group III (infertile patients with an index higher than 1.53 [n = 98]). There is no statistically significant difference in age, control group 39.4 ± 5.3 years, infertile Group I 35.8 ± 5.3 years, infertile Group II 34.5 ± 3.9 years, and infertile Group III 35.8 ± 7.4 years.

The results of sperm characteristics including HOST, motility, concentration, vitality, and sperm morphology were evaluated and compared between fertile group and infertile patients, as well as between the infertile groups (Table 1). Statistical analysis pointedly shows significant differences in the analysis of vitality, total and progressive motility, and in the percentage of immotile sperm of fertile men regarding to infertile men with normal indexes and with indexes above of the reference interval (Table 1).

Data from Table 1 also show that the comparison between infertile men, Group I presented higher values of sperm vitality than men from Group II ($p < 0.01$) and Group III ($p < 0.0001$). The differences were also significant for total and progressive motility, and immotile spermatozoa regarding Group II ($p < 0.01$) and Group III ($p < 0.0001$). Infertile patients with normal indices also

Table 1. Sperm characteristics in fertile and infertile men

Parameter	Fertile men (n = 36)	Infertile group I (n = 24)	Infertile group II (n = 44)	Infertile group III (n = 98)
Index range	1.23 to 1.53	Below 1.23	1.23 to 1.53	Higher than 1.53
Index average ± SD	1.38 ± 0.3	1.03 ± 0.2	1.37 ± 0.1	2.61 ± 1.5
Sperm count/mL	102.6 ± 80.8	45.7 ± 38.1*	84.0 ± 81.8*	65.5 ± 76.2*
Total sperm count	384.6 ± 307.1	156.3 ± 120.9*	239.8 ± 198.6*	207.5 ± 217.2*
Vitality	71.2 ± 10.3	78.1 ± 21.8†‡	61.1 ± 11.9‡§¶	48.8 ± 16.9**
Total motility	50.8 ± 9.9	55.3 ± 8.0†‡	41.2 ± 12.2‡§¶	27.1 ± 14.1**
Progressive motility	49.2 ± 10.1	53.5 ± 8.0†‡	39.2 ± 12.8‡§¶	24.9 ± 14.9**
Non progressive motility	1.0 ± 1.14	1.8 ± 1.1	2.0 ± 1.8	2.2 ± 1.8
Immotile	49.1 ± 9.9	44.7 ± 8.4†‡	58.8 ± 2.2‡§¶	72.9 ± 4.2**
Host	65.7 ± 9.5	57.2 ± 3.6*	54.0 ± 17.4§	52.0 ± 13.7§
Normal morphology	14.1 ± 5.4	8.6 ± 6.6§	12.0 ± 14.2*	6.7 ± 6.9§††
Small	8.6 ± 8.6	12.8 ± 12.5	10.0 ± 8.9	7.0 ± 7.7
Large	7.6 ± 5.2	7.0 ± 6.3	5.6 ± 5.7	5.0 ± 4.9
Pyriform	1.4 ± 1.0	0.7 ± 1.2	1.5 ± 2.3	0.9 ± 1.2
Amorphous	33.0 ± 8.4	29.0 ± 9.7	25.2 ± 8.4	27.8 ± 11.1
Rounded	0.1 ± 0.2	0.1 ± 0.3	0.1 ± 0.2	0.2 ± 0.6
Multi heads	0.2 ± 0.4	0.1 ± 0.3	0.1 ± 0.3	0.2 ± 0.5
Pin-headed	2.8 ± 2.0	5.1 ± 3.0	4.2 ± 3.8	4.5 ± 3.1
Tapered	1.1 ± 1.9	0.2 ± 0.4	1.0 ± 1.7	1.9 ± 2.9
Mid-piece defects	1.4 ± 1.6	1.5 ± 1.2	1.6 ± 1.9	0.9 ± 1.3
Tail defects	0.8 ± 1.2	1.1 ± 1.4	0.8 ± 0.9	1.1 ± 1.3
Combined anomalies	25.8 ± 7.2	29.9 ± 9.6	33.8 ± 12.4	37.8 ± 14.2
Lysed sperm	3.1 ± 2.2	3.9 ± 4.4	4.1 ± 4.1	6.0 ± 6.8

*p < 0.05 vs. fertile men.
†p < 0.01 vs. infertile Group II.
‡p < 0.0001 vs. infertile Group III.
§p < 0.01 vs. fertile men.
¶p < 0.001 vs. infertile Group III.
**p < 0.0001 vs. fertile men.
††p < 0.05 vs. infertile Group II.
HOST: Hypoosmotic swelling test; SD: standard deviation.

showed significant differences in vitality, total and progressive motility, and in the percentage of immotile sperm, compared to patients from Group III ($p < 0.001$).

The sperm count did present significant differences comparing fertile versus infertile men and between infertile groups ($p > 005$). On the other hand, sperm count/ejaculate presented significant differences in fertile versus infertile patients ($p < 0.05$) but when the infertile groups were compared; the differences were not significant ($p > 0.05$).

On the analysis of sperm morphology, this study detected significant differences of morphologically normal sperm in fertile group in relation to infertile men with normal indices ($p < 0.05$) and for infertile men with indices below and above reference ranges ($p < 0.01$). Significant differences were also observed Group II versus Group III ($p < 0.05$).

Interestingly, the differences were not significant in the HOST, when comparing the study groups of infertile men ($p > 0.05$). However, when infertile men were

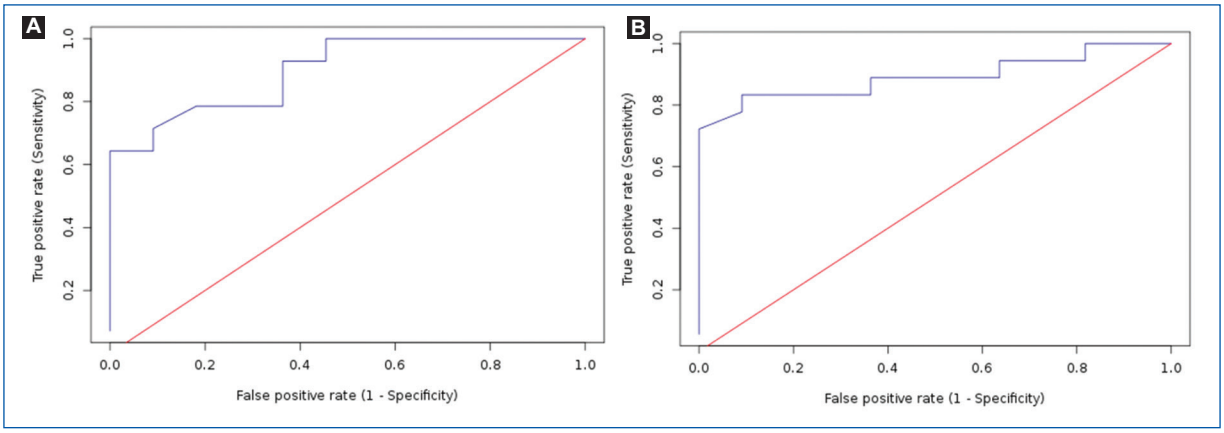

Figure 1. Receiver operating characteristics curve. Analysis of semen with abnormal sperm motility and normal morphology to evaluate epididymis function. The area under the curve (AUC) was 0.899 (95% for AUC 0.783-1) **(A)**. Abnormal sperm motility and morphology to evaluate epididymis plus testicle functions. The AUC was 0.891 (95% for AUC 0.77-1) **(B)**.

Table 2. Progressive motility and morphology in semen with an index higher than 1.53

Parameter	Poor motility and normal morphology (n = 26)	Poor motility and abnormal morphology (n = 36)	Normal motility and morphology (n = 26)
Progressive motility percentage	22.2 ± 8.6*	15.7 ± 9.8	44.9 ± 7.9
Sperm morphology percentage	9.83 ± 5.74	1.35 ± 0.88	14.5 ± 7.9
HOST percentage	52.5 ± 16.4	39.4 ± 16.9	73.9 ± 8.7
Index	2.98 ± 2.14	3.15 ± 1.19	1.87 ± 0.23

*Mean ± standard deviation.
HOST: hypoosmotic swelling test.

compared with fertile men, the differences were significant in all study groups ($p < 0.05$ for fertile versus Group I and $p < 0.01$ for fertile versus Group II and III).

Using ROC curve analysis, we shown that comparing Group I versus Group III, indices from 2.06 to 3.02 were indicative of disorders in epididymal function since they only presented poor sperm motility (area under the curve [AUC] 0,889 and 95% confidence interval [CI] 0.783-1) (Fig. 1a). On the other hand, comparing Group II versus Group III, indices from 2.60 to 4.91 were indicative of disorders simultaneously affecting epididymis plus testicles since they presented poor sperm motility and abnormal morphology (AUC 0.891, 95% CI 00.77-1) (Fig. 1b). Table 2 shows the results of the analysis of sperm characteristics in the study groups of this evaluation.

Discussion

The analysis of the sperm characteristics is pivotal to assess both sperm quantity and quality, whose normality is a basic requirement for conception[4,18,19]. This analysis is of the utmost importance, because it provides data about the testicular function, through the assessment of sperm production and of the morphological characteristics of the spermatozoa[20]. The HOST may also be considered a testicular marker because it evaluates the sperm membrane integrity[2], a sperm morphological characteristic whose development is an attribute of the spermatogenesis[21]. The HOST has also been considered a sperm function test since it assesses the fertilizing capacity of the spermatozoa *in vitro* and *in vivo*[10-14]. In the present study, the three groups of infertile men presented fewer values for HOST than the fertile men. These findings show clearly that the loss of the sperm membrane integrity is higher in infertile men than fertile men as reported previously[22-24].

In addition, the evaluation of the epididymis function is usually unexplored in both laboratory and clinical practice. Since the spermatozoa acquire progressive motility

during transit through the epididymis[25,26], the assessment of the sperm motility may be considered the only parameter with some ability for providing reports about the epididymis function in semen analysis routine.

The determination of neutral alpha-glucosidase in seminal plasma has also been recommended for investigating the epididymal function[27]. However, it has not been performed routinely, because it is expensive and time-consuming. As a consequence, the diagnosis of disorders affecting the epididymis through semen analysis is a huge trouble, chiefly of chronic epididymitis, which is a common cause of male infertility[9,28].

The present study is a proof-of-principle for a new index calculated by dividing the percentage HOST by the percentage of progressive motility provides valuable information about whether a disorder individually affects testicular and/or epididymal function. To the best of knowledge, this is the first attempt for assisting on the investigation of disorders affecting testes and/or epididymis singly. In general, semen analysis only investigates abnormal sperm characteristics (count, vitality, motility, and morphology), without providing information about which organ is affected.

Earlier studies have shown that low HOST usually correlates with poor sperm motility in infertile men[29]. However, this study showed that the greater the index, the lower the progressive motility, whereas the differences were not significant in the HOST when comparing the study groups of infertile men. Therefore, it is expected that indexes above the reference value might be indicative of abnormal epididymal function. In fact, table 1 shows that sperm motility presented significantly lower levels in infertile patients with an index above the reference levels. Interestingly, sperm vitality also showed significantly low levels in semen with higher indexes. On the other hand, these parameters did not present significant differences in patients with indices below the normal reference, although it would be expected that testicular dysfunctions would impair both sperm motility and vitality. These data suggest that poor vitality and motility in semen with higher index are seemingly indicative of a deficiency in the epididymis function.

At the same time, it was expected that lower indices would be indicative of deficiency in the testicular function. However, comparing fertile and infertile men, total sperm counts presented significantly lower levels in infertile patients with lower and higher indexes than the normal index values, which would also be indicative of deficiency in the testicular function in both (Table 1). Nevertheless, the differences were not significant when comparing the study groups of infertile patients. Likewise, the morphologically normal sperm presented significantly lower outcomes in semen with decreased and increased indexes. In addition, the outcomes of HOST were significantly higher in fertile patients than infertile patients.

Collectively, these outcomes show that abnormally low indexes indicate a deficiency in the testicular function, whereas abnormally high indexes indicate a deficiency in the epididymal function, especially in semen with moderate and severe asthenozoospermia. Nevertheless, in some instances, disorders affecting epididymal function may also be associated with testicular dysfunction, mainly in semen with moderate and severe teratoasthenozoospermia, according to data reported in table 2. This shows that disorders affecting the testes may simultaneously affect the epididymal function, like in varicocele men. Therefore, it may be assumed that they are independent functions and must be investigated separately. Clinically, this individual focus of investigation may be pivotal, for example, in severe asthenozoospermia and normal sperm morphology, which could be caused in some instances by chronic epididymitis[30]. Unfortunately, the investigation of this disorder usually is undervalued in the diagnostic workup of male infertility.

The present study observed that low indices do not indicate the presence epididymal dysfunction that higher indexes indicate a high likelihood of the presence of disorders affecting epididymis, which in some instances may be associated with abnormal testicular function. High indices would be able to detect disorders affecting epididymal function and disorders affecting both testicular and epididymal functions. Using ROC curves, the indices from 1.53 to 2.00 are inconclusive and sometimes even suggest normal functions of both (Table 2). However, indices from 2.06 to 3.02 are indicative of the presence of abnormal epididymal function alone when semen analysis detects moderate or severe asthenozoospermia and normal sperm morphology. Moreover, it was also observed that indices from 2.60 to 4.91 suggest a deficiency on both testicular and epididymal functions when outcomes of semen analysis show moderate or severe teratoasthenozoospermia. In addition, it was also observed that quite higher indices (≥ 5.00) are also detected in semen analysis routine.

The weakness of this study is that it is retrospective and based on data from laboratory practice of semen analysis and not on the diagnosis of disorders affecting testes and/or epididymes. However, it is expected that further researches will refine its clinical significance, as a new semen parameter for assisting the investigation of male infertility. Ultimately, it is simple, inexpensive, and can be performed in semen analysis routine of any laboratory.

Conclusions

The index proposed in the present study is not a parameter for diagnosis. However, it presumptively may determine whether a disorder affects epididymes and/or testicles in infertile men, which might thereby to be investigated using other available diagnostic tools. As discussed above, this may be particularly useful in patients with chronic epididymitis. In fact, semen with indexes from 2.06 to 3.02, moderate or severe asthenozoospermia, and normal morphology may be indicative of the presence of this disorder, especially in men with either unilateral or bilateral scrotal pain more than 3 months or longer in duration[30]. Likewise, antisperm antibody positivity can also be associated with chronic epididymal inflammation, with no testicular damage in infertile men[31]. At the same time, abnormally low indices are not indicative of abnormal function of the epididymis; this information is clinically relevant since the disorder solely would affects testicular function. Therefore, it may have a supplementary role for the work-up of male infertility in some instances.

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Conflicts of interest

The authors declare no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained approval from the Ethics Committee for analysis and publication of routinely acquired clinical data and informed consent was not required for this retrospective observational study.

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