Title of Manuscript: Pigments test strips: a rapid companion test to exclude sub-arachnoid haemorrhage

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Abbreviations

AU: Absorbance Units

COFRAC: COmité FRançais d'ACcréditation

CSF: CerebroSpinal Fluid

CT: Computed Tomography

Inc: Inconclusive

IQC: Interne Quality Control

ISAT: International Subarachnoid Aneurysm Trial

NBA: Net Bilirubin Absorbance

Neg: Negative

NPV: Negative Predictive Value

NOA: Net Oxyhemoglobin Absorbance

Pos: Positive

PPV: Positive Predictive Value

PTS: Pigments Test Strip-pads (urine test strips Multistix®, Siemens)

PTSb: Pigments Test Strip-pads for bilirubin detection

PTSh: Pigments Test Strip-pads for haemoglobin detection

PTShb: Pigments Test Strips-combined haemoglobin-bilirubin results

RBC: Red Blood Cells

SAH: SubArachnoid Haemorrhage

Se: Sensitivity

SEM: Standard error of the mean

Spe: Specificity

1. INTRODUCTION

Subarachnoid haemorrhage (SAH) accounts for 0.8 to 7% of all strokes [1]. In France, incidence ranges between 2.5 and 7/100 000 individuals [2]. The ISAT (International Subarachnoid Aneurysm Trial) study reported 25% of morbidity/mortality [3], which justifies immediate care and rapid diagnosis of SAH. An incorrect initial diagnosis of SAH worsens the prognosis [4]. SAH is a spontaneous arterial bleeding into the subarachnoid space. The main cause is the rupture of aneurysms (80%) [5]. Patients come to the emergency room with a sudden onset of severe headache, nausea, photophobia and neck pain. A high risk of complications exists, including symptomatic vasospasms, hydrocephalus and rebleeding. Early treatment set up of SAH prevents neurologic complications and functional impairments. The cerebral computed tomography (CT) scan is the gold standard method to diagnose SAH with 98-100% sensitivity within the first 6-12 hours [6]. However, 6 hours after the onset of symptoms, the sensitivity drops to about 86-93% [7] and it drops to approximately 50% after one week [8]. A cerebral angiography is usually performed on patients with a positive CT scan to confirm the presence and locate the aneurysm for preventive treatment of re-bleeding. In case of negative CT scan with clinical signs of SAH or beyond 12 hours after the first bleeding, a lumbar puncture of cerebrospinal fluid (CSF) is done to detect the abnormal presence of haem pigments (haemoglobin and bilirubin, a physiological metabolite of haemoglobin breakdown). They generally appear 2-12 hours after the bleeding started [9], and are indirect evidence of the bleeding. Indeed, after haemorrhage, red blood cells (RBC) are lysed and phagocyted by macrophages; they release oxyhaemoglobin further converted into bilirubin.

The presence of bilirubin in the CSF, also called xanthochromia, appears to be the most informative biomarker for suspected SAH [10]. By contrast, the presence of oxyhaemoglobin in

CSF may result from SAH or from *in vitro* haemolysis of red blood cells contaminating the sample due to traumatic puncture. Oxyhaemoglobin and bilirubin are usually detected by either visual inspection or spectrophotometry. Visual inspection is not reliable and should not be used because it only detects massive xanthochromia. [11]. Spectrophotometric methods are more sensitive and can detect the presence of oxyhaemoglobin and/or bilirubin by the measure of sample absorbances at known wavelengths. In the absence of French recommendations, we use the UK guidelines [10], recommending bilirubin rather than haemoglobin as the best biomarker for detecting SAH 12 hours after haemorrhage onset. Authors also proposed a helpful software to support the interpretation of CSF haem pigment analysis based on the UK guidelines [12]. They also describe the strict preanalytic conditions of sampling, including a large volume and rapid moderate centrifugation, and a fast light-protected transport to avoid misinterpretation.

The urine test strips (Multistix®) could be a quick and easy-to-use alternative to spectrophotometry to detect the presence of haemoglobin and bilirubin in CSF for laboratories that do not have a spectrophotometer or as a bedside diagnosis test. Interestingly, urine test strips require a small volume of sample, are available in most biochemistry laboratories for urine analysis, and present a very short turnaround time. Previous studies have already compared the performances of the Multistix® urine test strips for haemoglobin detection in CSF to the oxyhaemoglobin detection by spectrophotometry (at 415nm) or to RBC counts [13, 14]. However, the evaluation of bilirubin detection, the main haem pigment for SAH, was left out.

To help clinical management of SAH, the aim of this study was to compare the performance of the Multistix® urine test strips (named PTS in the manuscript) for haem pigments (bilirubin and haemoglobin) detection in CSF to the reference measure by spectrophotometry and to the definitive clinical SAH diagnosis.

2. MATERIALS AND METHODS

2.1. Study Design and Setting

This retrospective pilot study took place between 2014 and 2017 at Bordeaux university hospital. This work conforms in terms of the protection of personal health data and the protection of privacy to the framework provided by Article 65-2 of the Data Protection Act as amended and the General Regulation on the protection of personal data.

Data were collected from patient CSFs sent to the biochemistry laboratory for haem pigments detection (suspected SAH) by spectrophotometry. Net bilirubin absorbance (NBA) and net oxyhaemoglobin absorbance (NOA) were calculated as recommended by the UK guidelines. We systematically added the detection test of haem pigments by PTS and the determination CSF total protein concentration, in real time. Only the spectrophotometry results were communicated to the clinician. SAH suspicion according to the biomarkers presence was correlated to the final clinical SAH diagnosis (SAH was confirmed by CT imaging or cerebral angiography or excluded if there was another obvious cause or no evidence of SAH).

2.2 Methods

Pre-analytic conditions: samples were drawn at least 12 hours after the onset of symptoms, light-protected, and contained 15-20 drops. We performed systematic analysis of the last tube (the fourth). We centrifuged samples at 1500 g for 5 min. After analysis, they were stored at 4°C in the dark.

First, a drop of the centrifuged CSF sample was analysed on Multistix® urine test strips for bilirubin and haemoglobin search by a qualified laboratory technician who was blinded to the spectrophotometry test result. Bilirubin detection threshold is 0.4 mg/dL (7µmol/L) and

haemoglobin is positive when its concentration is above 0.015 mg/dL haemoglobin or when >5 RBC/µL are present. The Multistix® urine test strips semi-quantitative method was validated according to COFRAC (Comité Français d'accréditation) and ISO15189 norm requirements for urine matrix. The repeatability was conform for blood and bilirubin with 10 replicates on 3 different samples (100% sensitivity and specificity). We obtained a perfect reproducibility (100% sensitivity and specificity) for haemoglobin and bilirubin on 92 internal quality controls (including 1 negative and 1 positive control for each series) over a period of one and a half month. The exactitude has been assessed based on 4 different extern quality controls and was conform. We are registered with 2 External Quality Assessment Services: Probioqual (France) and RfB (Germany). We used 5 different Multistix® urine test strips lots for performances evaluation.

The sensitivity threshold of bilirubin detection in CSF with Multistix® PTS was assessed by determining of the limit of blank according to Clinical and Laboratory Standards Institute guidelines protocol EP17-2A. Three bilirubin-positive CSF samples were serially diluted to identify the limit of blank of PTS. Serial dilutions (from 0.10 AU to 0.02 AU) of 1 "home-made" internal CSF control quality tested 10 fold by PTS for each dilution were prepared. This PTS manual evaluation was carried out. A negative-bilirubin CSF sample (NBA = 0 AU) was performed simultaneously to facilitate the PTS reading. Each dilution value (NBA AU) was checked by the reference spectrophotometry. The spectrophotometer semi-quantitative method was validated according to COFRAC (French Quality committee) requirements for CSF biological matrix.

Then, we used a spectrophotometer Uvikon® Secomam in order to perform a spectrophotometric scan on the supernatant ranging from 350 to 600 nm by a qualified medical doctor blinded to

PTS results. Absorbance between 410 and 418 nm detects oxyhemoglobin and absorbance between 450 and 460 nm detects bilirubin [15]. The NBA (bilirubin) was calculated according to Chalmers' modification [16] of the original method [15]. Briefly, a tangent to the scan was drawn between 350 nm and 600 nm. NOA (oxyhaemoglobin) peak was measured from the predicted baseline around 415 nm and NBA (bilirubin) peak was measured from the predicted baseline around 476nm. When no oxyhaemoglobin peak was visible, NBA was adjusted to serum bilirubin and CSF total protein was determined. Our interpretation of the scans was based on NBA and NOA values expressed as absorbance units (AU) and according to UK Revised national guidelines [10]. Accordingly, a NBA>0.007 AU associated with a NOA>0.02 AU with a visible oxyhaemoglobin peak or an adjusted NBA>0.007 AU with CSF total protein <1g/L, were consistent with SAH (bilirubin + haemoglobin = positive) (10). If NBA≤0.007 AU with a NOA<0.1 AU or NBA>0.007 AU was associated with a NOA<0.02 AU with an adjusted NBA<0.007 AU, did not support SAH (bilirubin and haemoglobin undetectable = negative). Other cases were considered as inconclusive (bilirubin or haemoglobin alone). Qualified laboratory technicians and medical doctors were blinded to the study hypothesis. To ensure the quality of our results, 1 quality control (corresponding to a combined bilirubin-haemoglobin positive CSF) was analysed by spectrophotometry with each series of samples. Furthermore, our laboratory participated in the external quality evaluation program CSF haem pigments (UKNEQAS).

The CSF total protein levels were determined by a colorimetric method (Pyrogallol red) and the serum total bilirubin was determined by a colorimetric method (Diazonium salt) on Chemistry Analysers AU5400 (Beckman Coulter®).

2.3. Data Analysis

Sensitivity, specificity, positive predict value (PPV) and negative predictive value (NPV) of Multistix® urine test strips to support SAH were calculated using haemoglobin and bilirubin detection by spectrophotometry as the reference method (UK guidelines). Lab conclusions were compared to the final clinical SAH diagnosis.

Graph Pad software was used for statistical analysis. Descriptive data were expressed as mean+/- SEM (Standard Error of the Mean) and mean of differences with 95% confidence intervals. D'agostino & Pearson normality test determined normal distribution of the values. The two-tailed unpaired t-test were performed to compare means of two groups and calculate p values. p values less than 0.05 were considered statistically significant.

3.RESULTS

3.1 Study population

We analysed 136 CSF samples received in our laboratory for haem pigments analysis by spectrophotometry (with SAH suspicion). Of 136 CSF, 31 were excluded for absence of PTS results and 5 for absence of CSF total proteins concentration (Fig.1). Thus, 100 patients were included in the study. The analysed population included 42 males and 58 females, and patients were mainly from the emergency room. Age group distribution reflected that SAH suspicion were more frequent in 36-50 years old patients. There were 19/100 biological confirmation of SAH suspicion using the UK algorithm and 13/100 were clinically diagnosed in our global population (N=100).

3.2 Pigments test strips biological performance for haem pigments detection

First, we evaluated the sensitivity, specificity, NPV and PPV of haemoglobin or bilirubin by PTS *versus* spectrophotometry detection in 100 CSF samples. Indeed, spectrophotometry is the current reference method to detect haem pigments in CSF. The main objective for this biological test was to eliminate a SAH. Haem pigments detection required high sensitivity and high NPV to avoid false negative results.

Pigments test strip-pads for haemoglobin detection (PTSh) displayed a perfect sensitivity (1.00, 95% confidence interval (CI) 0.88 to 1.00) and a low specificity (0.47, 95% CI 0.36 to 0.58) (Table 1). The perfect NPV (1.00, 95% CI 0.90 to 1.00) of PTSh was technically interesting. To confirm this high performance, we compared the NOA performance within the haemoglobin negative and positive PTSh. NOA mean in the haemoglobin positive PTSh samples (0.114±0.028 AU) was greatly higher than in the haemoglobin negative PTSh samples (undetectable AU, mean of differences 0.114 AU, 95% CI 0.037 to 0.191, p=0.004, Fig.2A).

For bilirubin detection, recommended as the best biomarker by the UK Revised national guidelines, pigments test strip-pads for bilirubin detection (PTSb) showed a modest sensitivity (0.54, 95% CI 0.35 to 0.72) but a good specificity (0.97, 95% CI 0.91 to 1.00) (Table 2). PTSb was less sensitive than spectrophotometry (lower NPV). PTSb threshold was probably higher than that of spectrophotometry. To test this hypothesis, we compared the NBA performance within the bilirubin negative and positive PTSb. NBA mean in the bilirubin positive PTSb samples (mean 0.131±0.025 AU) was 5-fold higher than in the bilirubin negative PTSb samples (mean 0.025±0.004 AU, mean of differences 0.106 AU, 95% CI 0.054 to 0.157, p=0.0003, Fig.2B). The lowest AU value for positive PTS was 0.03, suggesting that the threshold positive value for PTSb was around 0.03. To confirm this result, we diluted 3 bilirubin positive CSF

samples and found a positivity threshold at 0.034±0.006 AU (Spectrophotometry reproducibility coefficient of variation =17%) for PTSb (Fig.2C) whereas the expected AU by spectrophotometry was 0.007. In accordance with international guidelines, the cut-off of PTSb was confirmed by the internal quality CSF control, which identified the limit of blank at 0.03 AU and a positive threshold (limit of detection) of the PTSb at 0.04 AU (Fig 2C). This result explained the lack of sensitivity of PTSb.

Together, these data suggest that PTSh was more sensitive than spectrophotometry and that spectrophotometry was more sensitive than PTSb.

Because both bilirubin and haemoglobin presences are required to ascertain biological SAH as to UK algorithm guidelines by spectrum, we next considered positive pigments test strip-combined haemoglobin-bilirubin results (PTShb) when bilirubin and haemoglobin were detected. The PTShb was negative when haemoglobin and bilirubin were undetectable. The PTShb was reported as inconclusive when only haemoglobin or bilirubin was present. So, we interpreted the haem pigments results between PTShb and spectrophotometry using the UK guidelines. Then, as our main goal was to evaluate the ability of the PTShb result to rule out SAH (high sensitivity and NPV), we defined those inconclusive results as positive for the 2 tests (PTShb and spectrophotometry). This resulted in high sensitivity (0.97, 95% CI 0.83 to 1.00) and modest specificity (0.46 95% CI 0.35 to 0.57) (Table 3). These results suggest that the combined presence of haemoglobin and bilirubin increases PTShb sensitivity.

3.3 Pigments test strips performance for SAH diagnosis exclusion

As PTS, spectrophotometry detects biomarkers as a sign of potential SAH. To fully evaluate PTShb, it was necessary to reposition the results in light of the final clinical SAH diagnosis. To

evaluate the clinical performance of our test to rule out SAH diagnosis, we analysed the concordance between the negative PTShb (undetectable bilirubin and haemoglobin) or the negative spectra using the UK guidelines and the final clinical SAH diagnosis. Here again, we considered detectable haemoglobin and/or bilirubin as positive. Thirteen out 100 patients were clinically confirmed for SAH. PTShb was less specific than the spectrum. Interestingly, all clinical SAH had a positive or inconclusive PTShb. However, PPV was low due to many false positive or inconclusive results (54/67 patients, Table 4) versus 18/31 patients with spectrum, indicating that PTShb, like spectrum, was not a good test for SAH biological diagnosis. When PTShb was negative for haem pigments, none of the patients (0/33) presented confirmed clinical SAH (PTShb sensitivity = 1.00, 95% CI 0.77 to 1.00, Table 4) while there was 1/70 false negative for spectrum analysis (spectrum sensitivity = 0.92, 95% CI 0.67 to 1.00, Table 4). Finally, although less specific, PTShb sensitivity was at least as good as that of the spectrum, with a perfect NPV.

Taken together, these results suggest that an undetectable bilirubin and haemoglobin by PTShb could probably rule out clinical SAH when spectrophotometry was not available.

4. DISCUSSION

In this study, we evaluated the Multistix® urine test strips as a rapid diagnosis tool in clinical suspicion of SAH.

Although haem pigments analysis by Multistix® urine test strips was not recommended by the UK published guidelines [10], our results showed good performance to exclude SAH. Spectrophotometers are not always available and many laboratories currently use PTS instead for SAH-derived biomarkers detection in CSF samples when SAH is suspected. Multistix® urine

test strips are available in most laboratories, easy to run as a bedside diagnosis test, need low volumes of CSF and give quick results. As Multistix® urine test strips are already used and could be helpful for SAH clinical management, their performance evaluation was required.

We systematically blindly analysed the presence of pigments in CSFs by both Multistix® urine test strips and spectrophotometry.

First, we analysed PTSh and PTSb separately, as compared to spectrophotometry. We confirmed that PTSh was very sensitive [13,14] but PTSb was not suitable for bilirubin detection because the colour change remained difficult to assess and PTSb detected only macroscopic xanthochromia. Lack of sensitivity led to false negative results for bilirubin detection when the NBA was below 0.04 AU. According to the UK guidelines, the critical threshold for probable SAH is a NBA>0.007 AU (almost 5 times less). So, despite its simplicity PTSb, did not seem to be reliable. However, when we considered PTShb as compared to the spectrophotometry algorithm, we obtained a high sensitivity but a modest specificity. These results supported that PTShb was nearly as sensitive as spectrophotometry using the UK guidelines to rule out SAH. Finally, and importantly, when we compared PTShb to clinical diagnosis, we found that PTShb biological performance had a perfect sensitivity (no false negatives) for SAH exclusion. However, positive PTShb could not affirm SAH because of the numerous false positives (54/67, PPV = 0.19). Noticeably, spectrophotometry also could not be used, with 18/30 false positives (PPV = 0.40). Indeed, this low PPV was also described by Birch *et al* [17] where only 0.38 of positive spectra had a clinically confirmed SAH. These results may explain the discordance between PTShb performance compared to the recommended spectrophotometry and final clinical SAH diagnosis. So, the PTShb alone could rule out SAH but should not be used to confirm SAH.

Others studies were interested in SAH diagnosis exclusion by alternative biological tools. Birch

K *et al* [17] have already highlighted that SAH is associated with higher CSF total protein concentrations and concluded that no single CSF total protein value could be used to rule out SAH. Other biomarkers such as RBC counts had a theoretical lower limit of detection than that of the Multistix® urine test strips threshold (1 RBC *vs* 5 RBC/µL). However, it was imperfect because this approach did not differentiate SAH diagnosis from traumatic lumbar punctures in first tube. An absolute RBC count < 500 cells in tube 4 (manually measured) could exclude SAH [18]. The major limit was that, in our hands, the collection of 4 tubes, numbered in the order of sampling, is rare. Finally, other biological tools were tested such as CSF siderophages count, CSF ferritin concentration and CSF methaemoglobin, but seemed to lack sensitivity for SAH diagnostic [19]. Because lumbar punctures are invasive, peripheral blood biomarkers were tested. High levels of TNF-alpha receptor 1 (TNFR1) in venous blood were associated with the presence of aneurysms in case of SAH [20]. Similarly, combination of 3 microRNAs was upregulated during the SAH in peripheral blood and could help monitoring the different phases of SAH [21]. However, these markers are not routinely determined in medical laboratories.

None of these biomarkers could easily exclude SAH and pigments detection in CSF was still the best biomarker.

Our study demonstrates that Multistix® urine test strips could be an alternative to spectrophotometry. However, our study presents some limitations. It is a descriptive study where haem pigments detection was usually prescribed to exclude SAH diagnosis, but in a few cases, it was used for SAH patients follow-up. Moreover, haem detection analysis is a second-line test when CT-scan is negative after 12 hours SAH symptoms. This is a strict condition to use haem pigments presence in CSF. Another limitation was that the urine tests strips were read by

operators, which may deteriorate the performance. Automated reading should be preferred to limit this important source of fluctuations.

Finally, this monocentric study included a limited number of patients since it needed Multistix® (Siemens) urine test strips limited the generalizability of the results. Bigger cohorts are needed to confirm this promising result. Other urine test strip tools should also be used to check if they present similar performance.

5. CONCLUSIONS

In conclusion, 2 to 3 percent of SAH have a negative cerebral CT scan within the 12 hours following the start of haemorrhage, and CT-Scan sensitivity decreases after 12 hours. CSF haem pigments absence can help to rule out SAH. The reference method for lab-based SAH exclusion is spectrophotometry. PTShb negativity determined by Multistix® urine test strips appeared to be a good first-line, easy and bedside test to rule out SAH diagnosis. However, spectrum analysis must remain the reference method when available.

Table 1: Pigments test strip *vs* spectrum for haemoglobin detection

		PTSh (N=100)	
		Negative	Positive
Spectrum	Negative	34	39
oxyhaemoglobin	Positive	0	27
Total		34	66
Sensitivity		1.00	
Negative predictive value		1.00	
Specificity		0.47	
Positive predictive value		0.41	

PTSh: Pigments test strips-pads for haemoglobin detection.

Table 2: Pigments test strip *vs* spectrum for bilirubin detection

		PTSb (N=100)	
		Negative	Positive
Spectrum bilirubin	Negative	74	2
	Positive	11	13
Total		85	15
Sensitivity		0.54	
Negative predictive value		0.87	
Specificity		0.97	
Positive predictive value		0.87	

PTSb: Pigments test strips-pads for bilirubin detection.

Table 3: Pigments test strip *vs* spectrum for biological SAH detection

		PTShb (N=100)		
		Negative	Positive	Inconclusive
Biological SAH by spectrum	Negative	32	0	38
	Positive	1	12	9
	Inconclusive	0	2	6
Total		33	14	53
Sensitivity			0.97	
Negative predictive value			0.97	
Specificity			0.46	
Positive predictive value			0.43	

PTShb: Pigments test strips-combined haemoglobin-bilirubin results, SAH: Subarachnoid haemorrhage.

Table 4: Pigments test strip or spectrum *vs* SAH final clinical diagnosis

		PTShb (N=100)			**Biological SAH by spectrum** (N=100)		
		Neg	Pos	Inc	Neg	Pos	Inc
Clinical SAH diagnosis	Neg	33	7	47	69	12	6
	Pos	0	7	6	1	7	5
Total		33	14	53	70	19	11
Se		1.00			0.92		
NPV		1.00			0.99		
Spe		0.38			0.79		
PPV		0.19			0.40		

Inc: Inconclusive, Neg: Negative, NPV: Negative predictive value, Pos: Positive, PPV: Positive predictive value, PTShb: Pigments test strips-combined haemoglobin-bilirubin results, SAH: Subarachnoid haemorrhage, Se: Sensitivity, Spe: Specificity.

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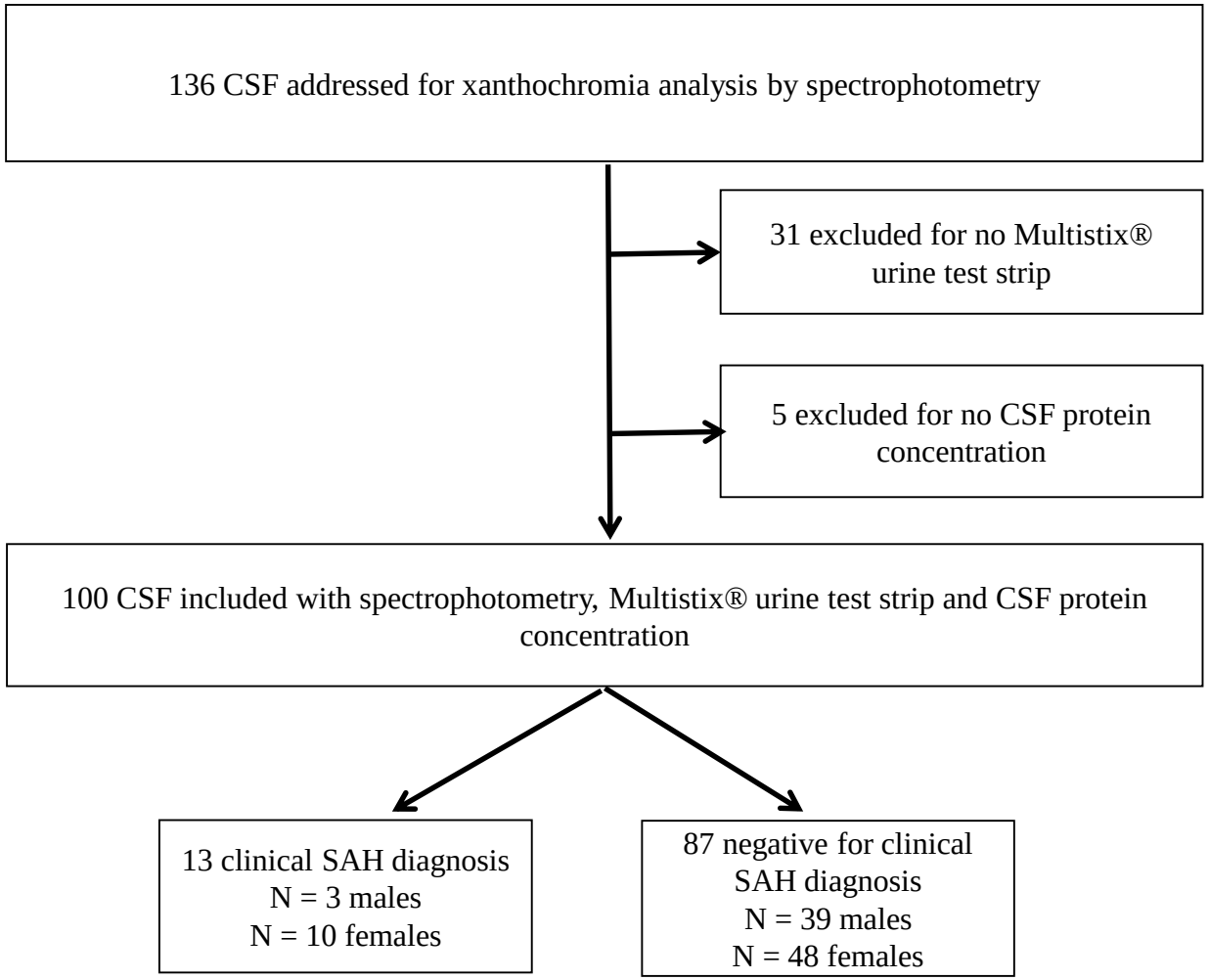
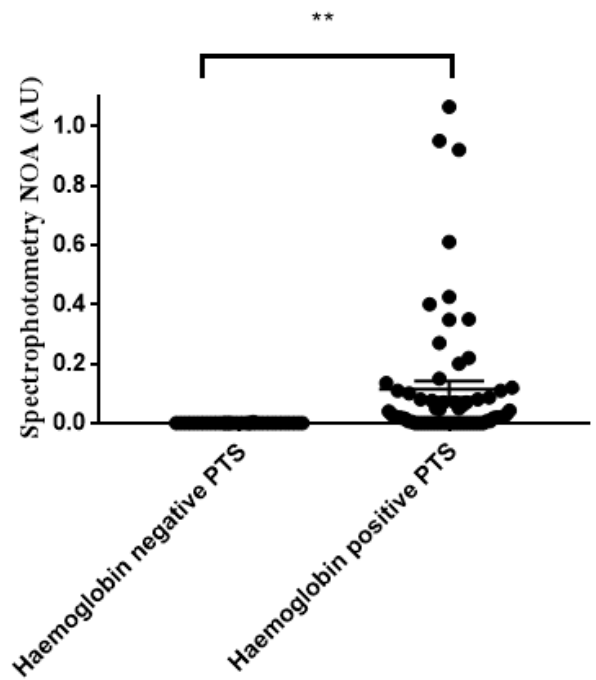

136 CSF addressed for xanthochromia analysis by spectrophotometry
31 excluded for no Multistix® urine test strip
5 excluded for no CSF protein concentration
100 CSF included with spectrophotometry, Multistix® urine test strip and CSF protein concentration
13 clinical SAH diagnosis
N = 3 males
N = 10 females
87 negative for clinical SAH diagnosis
N = 39 males
N = 48 females

A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Spectrophotometry NOA (AU)
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Haemoglobin negative PTS
Haemoglobin positive PTS

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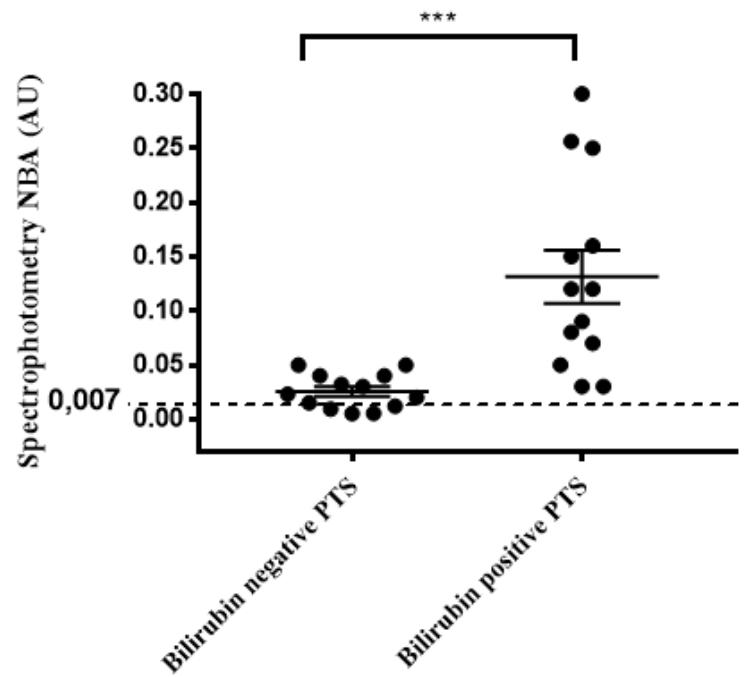
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Spectrophotometry NBA (AU)
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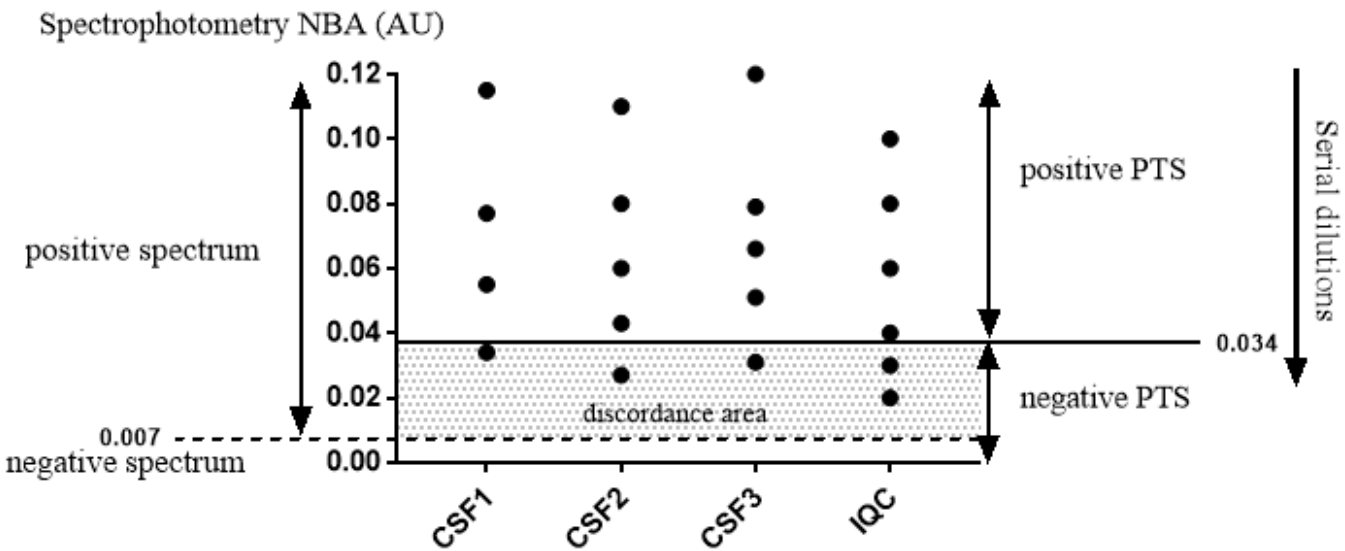
Bilirubin negative PTS
Bilirubin positive PTS

C


Spectrophotometry NBA (AU)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
positive spectrum
0.007
negative spectrum
discordance area
positive PTS
negative PTS
0.034
Serial dilutions
CSF1
CSF2
CSF3
IQC