INTRODUCTION

The term stroke is a broad term used for incidents associated with thrombosis, hemorrhage, and embolism. In the Western world there has been a sharp increase in stroke and thrombotic events, and acute stroke is one of the leading factors of morbidity and mortality worldwide. Stroke is also the number three cause of death in industrialized countries. Approximately 795,000 Americans suffer a new or recurrent stroke each year [1]. Stroke is also classified as the most important cause of morbidity and long-term disability in Europe and it imposes an enormous economic burden there as well [2].

Ischemic stroke accounts for about 87% of all stroke cases. Also, approximately 80% of embolism-related deaths are from stroke and 20% from other systemic thrombo-embolisms [3]. Many ischemic strokes occur without a well-defined etiology and are known as cryptogenic stroke [4]. A thrombotic event is associated with a change in hemostasis and cellular components that play a fundamental role, here including blood platelets and fibrin networks. Altered fibrin clot structure and resistance to fibrinolysis is associated with cryptogenic, as well as other ischemic strokes [5]. The structure of the fibrin clot, which can be characterized in terms of a branched network, directly affects the clot’s fibrinolytic and visco-elastic properties [6].

Currently, the key to advancing the treatment of stroke and improving primary and secondary prevention is enhanced public and health practitioner awareness. Existing diagnosis of stroke relies on physician clinical examination and is further supplemented...
with various neuro-imaging techniques [7]. One of the
criteria used for determination of the subtype of isch-
emic stroke is the original TOAST criteria (Trial of ORG
10172 in Acute Stroke Treatment) [8]. Also, the WHO
also has a set of criteria to be used when identifying
ischemic stroke [9,10].

Various biological markers are also available to
study stroke, however, with varying levels of accuracy.
Saenger and Christenson in 2010 published a very
comprehensive review regarding available tools in
pathology to identify and predict different thrombotic
events [7]. They mentioned that clinical diagnoses are
predicated on obtaining an accurate medical history
and a thorough physical assessment of the patient
during the critical window of opportunity (which is only
a few hours). This can be done in conjunction with the
42-point National Institutes of Health Stroke Scale that
was developed and designed to be completed within
5–8 min [11, 12]. However, neuro-imaging (NCCT or
MRI) remains one of the most useful tools, together
with electrolyte, electrocardiogram, blood and plate-
et analysis, as well as cardiac markers such as tro-
ponin [7]. Nevertheless, there is a need for additional
laboratory-based diagnostic tools, because of a lack of
accurate diagnostic criteria based on specific biomark-
ers. Cerebral infarction biomarkers have the potential
to alter and expedite the differential diagnosis and
prediction of stroke, particularly where neuro-imaging
results are doubtful [7]. However, Saenger and
Christenson noted that information from this avenue
is not that useful, because of the slow release of glial
and neuronal proteins across the blood–brain barrier
after stroke and the lack of diagnostic specificity and
also sensitivity.

Currently there is no tool to aid in possible stroke
risk assessment. Here, we investigate the possibility for
an additional, qualitative morphological tool to pre-
dict thrombotic stroke. Previously, it was shown that
patients with high risk factors, such as smoking and
pregnancy, as well as patients 48 h after stroke, show
a changed fibrin network, using a scanning electron
microscope (SEM) [13]. We suggest, therefore, that when
a high-risk population is screened using fibrin mor-
phology as screening tool, ultrastructure could be used as
an accurate predictor for future thrombotic events. A
SEM procedure that is to be used as a general screening
tool must be relatively cheap and accessible. Typical
research SEMs are extremely expensive and available
only at research institutions. Here we investigate the
use of a smaller, desktop model SEM to study fibrin
networks in early detection of stroke risk. We compare
micrographs from this desktop machine to that of high-
resolution research SEM micrographs from a group of
patients 48h post-stroke. This was done to determine
the quality of the micrographs on the smaller model.
A desktop model may be more accessible to pathology
laboratories and may therefore be more suitable for
general screening purposes.

MATERIALS AND METHODS

Equipment Used

A Zeiss ULTRA plus FEG-SEM with InLens capabilities
was used to study surface morphology and micrographs
were taken at 1 kV. Also a desktop portable ZEOL SEM
(ZEOLNeoScope) was used to reevaluate the samples.
The working distance of this machine is 2 mm, apert-
ture is 20 µm, and scanning speed is 128 s for purpose
of micrograph. The Zeiss instrument is located in the
Microscopy and Microanalysis Unit of the University of
Pretoria, Pretoria, South Africa. The JEOlNeoScope was
kindly loaned to us by Advanced Laboratory Solutions,
South Africa. Micrographs were taken at 10 kV, work-
ing distance of 10 mm, aperture of 20 µm, and scanning
speed of 80 s.

Sample

In our laboratory, we have a SEM bank of thousands
of fibrin network micrographs of healthy individuals.
Six controls were chosen from this bank for this study
and their morphology is in accordance with control
samples in the bank. Citrate blood samples were col-
lected 48 h post-stroke from 20 ischemic stroke patients
(ethical clearance was obtained from the Research
Ethics Committee, Faculty Health Sciences, University
of Pretoria, which complies with ICH-GCP guidelines
and has U.S. Federal-wide Assurance). Exclusion cri-
tera were acute illness, cancer, and hepatic or renal
dysfunction, and the diagnosis was done by a neu-
rologist according to the TOAST criteria. All patients
underwent magnetic resonance (MR) brain scanning
(which confirmed the ischemic stroke and excluded
all other causes) and standard thrombophilia screen-
ing. A standard stroke workup was done, including
cardiac and carotid ultrasonography, ECG, chest x-ray,
and blood tests. Data concerning demographics and
general health status were collected using a question-
aire, and smoking was defined as smoking at least 5
cigarettes per day.

Preparation of Fibrin Clots

Citrated Whole Blood with the Addition of
Thrombin

Fresh platelet-rich plasma (PRP) was prepared by
centrifuging citrated blood at 1000 rpm (maximum
RCF = 17.523 × g; 1250g) for 2 min. Human thrombin
(provided by the South African National Blood Service)
was used to prepare these fibrin clots from the donor.
The thrombin solution was at a concentration of 20 U/
mg and was made up in a biological buffer containing
0.2% human serum albumin. When thrombin is added
to PRP, fibrinogen is converted to fibrin and intracellular
platelet components, e.g., transforming growth factor, platelet-derived growth factor, and fibroelastic growth factor, are released into the coagulum. Ten microliters of the PRP was mixed with 10 µL of human thrombin on a 0.2-µm Millipore membrane to form the coagulum (fibrin clot).

Preparation of Washed Fibrin Clots for SEM
Washed fibrin clots were fixed in a 2.5% glutaraldehyde/formaldehyde in Dulbecco's phosphate-buffered saline (DPBS) solution with a pH of 7.4 for 30 min. Each clot was rinsed three times in phosphate buffer for 5 min before being fixed for 30 min with 1% osmium tetroxide (OsO₄). The samples were rinsed 3 times with PBS for 5 min and were dehydrated serially in 30, 50, 70, and 90% and 3 times with 100% ethanol. The material was critical point dried, mounted, and coated with carbon.

RESULTS AND DISCUSSION
In their comprehensive review, Saenger and Christenson (2010) discussed the current available biomarkers to detect stroke (summarized in Table 1) [7]. Although Table 1 shows the biomarkers in stroke with potential, currently there are no known individual biomarkers for routine use in either acute stroke diagnosis or prediction of risk. The authors also discuss and list miscellaneous biomarkers used in strategies for stroke diagnosis, but these are not discussed further here.

Stroke diagnosis remains predominantly dependent on clinical interpretation, with little potential currently for discovery of stroke biomarkers, although such a biomarker would greatly improve patient care. However, prediction of stroke will have an important impact on the health care system. Here, we investigated the potential for such a tool, at a different level than the traditional biomarkers. Ultrastructure and morphology might provide a cheap, reliable, and very accessible avenue of stroke prediction and tracking the stroke patient's recovery and long-term treatment regimes. Pretorius and co-workers have previously shown that platelet and fibrin morphology change in disease conditions like cancer, HIV/AIDS and anti-inflammatory conditions, e.g., asthma [14, 15]. Changed morphology was also shown in humans 48 h following stroke [13]. It is therefore hypothesized that when a high-risk population is screened using fibrin morphology as screening tool, ultrastructure could be used as an accurate predictor for future thrombotic events.

During the coagulation process, fibrin arranges into networks due to the coagulation cascade. These networks consist of major, thick fibers (thick, white arrow) and minor, thin fibers (thin, white arrows), dispersed in between them (Figure 1). However, in the presence of disease or unhealthy life style, the coagulation process may be impaired or changed. These changes may be studied in the laboratory by adding thrombin to platelet-rich plasma. If the factors governing “normal” coagulation potential with the possibility to coagulate into normal fibrin networks are changed in the platelet-rich plasma, this will cause the fibrin to form a visibly changed coagulated morphology. These changes may be seen long before an actual event. Examples of such changes are prevalent in, e.g., smokers [16]. Figure 2 shows representative micrographs of 6 of the patients 48 h after stroke. Here the typical fibrin network, consisting of major, thick fibers with finely dispersed minor, thin fibers is changed and a matted layer of fibrin is seen after activation with thrombin.

Figure 3 shows a control fiber network using the desktop ZEOLNeoScope SEM. Although magnification

TABLE 1 Biomarkers with possible use, indicating stroke (summarized from Saenger and Christenson*"

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Role during stroke</th>
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<tbody>
<tr>
<td>Lipoprotein-associated phospholipase A2 (Lp-PLA2)</td>
<td>Independent inflammatory marker of cardiovascular risk</td>
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<tr>
<td>Asymmetric dimethylarginine (ADMA)</td>
<td>Plasma levels correlate with stroke risk</td>
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<tr>
<td>Matrix metalloproteinases (MMPs)</td>
<td>In stroke MMP mediated proteolysis causing blood–brain barrier leakage</td>
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<tr>
<td>S-100 β</td>
<td>Glial protein that is increased following stroke</td>
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<tr>
<td>N-Methyl-D-aspartic acid (NMDA) receptors</td>
<td>Glutamate neurotransmitter and may be important as stroke biomarker</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Specific to astrocytes and might have potential as biomarker</td>
</tr>
<tr>
<td>Park7 (or DJ-1)</td>
<td>Oncogene and autosomal recessive gene related to Parkinson disease. Potential as biomarker needs to be investigated.</td>
</tr>
</tbody>
</table>

FIGURE 1 Fibrin network created by adding thrombin to platelet-rich plasma of a healthy volunteer. The micrograph is representative of the typical morphology of a healthy individual and represents the typical ultrastructure of the control bank, which consists of thousands of micrographs of healthy individuals. This micrograph was viewed with Zeiss ULTRA plus FEG-SEM. Thick, white arrow = major, thick fibers; thin, white arrows = minor, thin fibers. Scale = 1 µm.
on this machine is much lower than that of the research machine, enough detail is visible to conclude that micrographs show relevant structural morphological information. Figure 4 shows the same 6 stroke patients shown in Figure 2, but viewed with the desktop portable ZEOL SEM. Thick, white arrows show fibers forming matted fibrin plates and thin, white arrows show small isolated areas of fibers organized similar to that of controls. Although the quality of the micrographs is arguably better on the research machine, information needed to judge whether a patient is at risk is adequately visible. This machine costs approximately 60,000 euros versus the more expensive research SEM machines that might cost in excess of 300,000 euros. This desktop version produces micrographs where the same tight fibrin networks are visible, and it is suggested that the quality of the micrographs are high enough to be used as a general, qualitative screening tool. Using such a desktop SEM, a fibrin profile for a patient can be generated and used as general screening tool among a population with potential risk. This
A technique might be used to identify individuals at risk, long before the actual event. It may, e.g., be used to study individuals at risk in old-age homes, individuals with a family history of stroke, or obese patients. Also, such a tool may provide valuable information in recovering stroke patients, where the medical practitioner wants to follow up whether the medication has aided the return of the fibrin network to that of a normal profile. Here, it is not suggested that this should replace the currently used techniques, but may be valuable as additional instrument.

**CONCLUSION**

SEM analyses of fibrin networks may have the potential to act as general screening tool to identify patients that might be at risk. Because of the nature of the analysis, the screening may be done on a portable desktop SEM like the ZEOLNeoScope, which possesses magnification capabilities of up to 20,000× magnification. Although this machine does not have the magnification possibilities of the bigger SEM, high magnification is not required to come to a conclusion regarding the general architecture of the fibrin network. Concerns that might be raised when using this as tool, may be adequate training of user, as well as a standardized method to scan and grade the clots, to determine the presence of abnormal fibrin morphology present in the total surface of the clot. Additional studies using morphometric techniques that objectively analyze fiber thickness may also be of value. Therefore, such a machine may typically be used in a pathology laboratory, where the cost of the machine will be on par with the other equipment used.
Also, laboratory-qualified technical personnel will be able to do such analysis with a few hours of training. Such a screening tool may perhaps in the future be used in conjunction with the various available biomarkers or radiographic tools (like MRI analysis, in the case of identification of stroke) and potentially may provide a method of preventing this very debilitating disease.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES