Molecular diagnostics for sleeping sickness: what is the benefit for the patient?

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Sleeping sickness, or human African trypanosomiasis, is a vector-borne disease caused by two subspecies of the protozoan parasite *Trypanosoma brucei*, and is geographically restricted to sub-Saharan Africa. Although the disease causes major public-health and socioeconomic problems among affected populations, sleeping sickness is one of the world's most neglected diseases. Within the rapidly evolving field of biotechnology, many molecular diagnostics have been developed to detect the parasite. These range from conventional, high-tech, and low-tech PCR formats (eg, isothermal nucleic-acid-amplification techniques), to direct visualisation of the parasite's nucleic acids by fluorescent probes. Besides reviewing the most important molecular diagnostics available, we discuss their current role in diagnosis and disease control. Although powerful, molecular diagnostics are confined to research settings and do not reach the patient or national control programmes. The current formats are not applicable to field conditions, and simplification, standardisation, and proper test evaluation in the target setting should be the main focus for future development.

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Introduction

Human African trypanosomiasis, also known as sleeping sickness, is caused by trypanosomes belonging to the species *Trypanosoma brucei*.[1] Two subspecies, *T brucei gambiense* and *T brucei rhodesiense*, can cause human disease and are cyclically transmitted by tsetse flies. Because the disease is exclusively associated with tsetse fly habitats, it is restricted to sub-Saharan Africa and is usually found in remote rural areas. *T brucei gambiense* sleeping sickness is endemic in west and central Africa, whereas *T brucei rhodesiense* sleeping sickness is restricted to east Africa. The former is responsible for most of the reported cases, and human beings are believed to be the main host.[2] By contrast, *T brucei rhodesiense* infection is a zoonosis with cattle and wild animals as the main reservoir and human beings as accidental hosts.[2]

According to a recent epidemiological update by WHO,[3,4] 10 000–40 000 new *T brucei gambiense* sleeping sickness cases were annually reported between 1997 and 2006, with Democratic Republic of the Congo, Angola, and Sudan the most affected countries (>1000 new cases per year). Between 450 and 750 *T brucei rhodesiense* sleeping sickness cases were reported annually in the same period, with Uganda and Tanzania as the leading countries.[3,4] The actual number of patients with sleeping sickness is difficult to estimate because, in many foci, few and irregular control activities, if any, are being implemented.

Sleeping sickness is a fatal disease if untreated and progresses along two phases. After infection by a bite of an infected tsetse fly, parasites multiply in the blood, lymph, and peripheral tissues of the patient. After this haemolymphatic phase, parasites can cross the blood–brain barrier and invade the CNS. In the neurological phase, the patient shows the signs and symptoms associated with sleeping sickness, such as mental changes, neurological disorders, and disturbance of the sleep–wake cycle, which gives the disease its name.

The challenge of accurate diagnosis

Because the available drugs to treat sleeping sickness are either toxic or cumbersome to administer, and because symptoms are generally not specific enough to start treatment, accurate diagnostic tests are needed to decide who to treat and how to treat them. In areas of *T brucei gambiense* sleeping sickness, control programmes systematically screen the population in villages at risk. All inhabitants are screened for swollen cervical lymph nodes (Winterbottom's sign) or for the presence of specific antibodies against the parasite with the card agglutination test for trypanosomiasis (CATT).[5] Because this screening method is not 100% specific, infection should be confirmed by detection of the parasite in the lymph or blood. Additionally, the sensitivity of the CATT is rather low in several foci in west Africa, which might be explained by the absence of the gene that encodes the variant surface glycoprotein LiTat 1.3 in some *T brucei gambiense* strains.[6]

Microscopic examination of lymph aspirated from enlarged cervical lymph nodes or of blood films in *T brucei rhodesiense* endemic areas are usually the first tests done because of their simplicity. Sensitivity of parasite detection on blood can be increased by prior concentration steps such as the microhaematocrit centrifuge technique (mHCT),[7] quantitative buffy coat analysis,[8] and the mini-anion-exchange centrifugation technique (mAECT).[9] The latter technique is the most sensitive, enabling the detection of about 100 trypanosomes per mL blood.[10]

Confirmed patients and individuals without parasitological confirmation but with convincing neurological symptoms or a high CATT end titre then undergo lumbar puncture for microscopic examination of the cerebrospinal fluid. The neurological phase of sleeping sickness is diagnosed by the detection of parasites or by an increased white-blood-cell count in the cerebrospinal fluid.[11,12] The white-blood-cell threshold above which a specific treatment for the neurological phase should be given is still under debate. Most national

	Target group	Target	Assay	Reported detection limit per reaction (parasites [n])*
Moser et al[24]	*Trypanozoon*	Satellite DNA	Single PCR	0·1
Kabiri et al[25]	*Trypanozoon*	ESAG6/7	Nested PCR	0·1
Holland et al[26]	*Trypanozoon*	ESAG6/7	Single PCR	5
Desquesnes et al[27]	*Trypanozoon*	ITS1 rDNA	Single PCR	100–10 000†
Njiru et al[28]	*Trypanozoon*	ITS1 rDNA	Single PCR	100–1000
Cox et al[29]	*Trypanozoon*	ITS1 rDNA	Nested PCR	1
Adams et al[30]	*Trypanozoon*	ITS1 rDNA	Nested PCR	1
Hamilton et al[31]	*Trypanozoon*	18S and 28Sα rDNA	FFLB	10
Morrison et al[32]	*Trypanozoon*	ITS1 and satellite DNA	MDA and PCR	2·5 (ITS1) and 0·025 (satellite DNA)
Becker et al[33]	*Trypanozoon*	Satellite DNA	Real-time PCR	0·1
Deborggraeve et al[34]	*Trypanozoon*	18S rDNA	PCR oligochromatography	0·05
Kuboki et al[35]	*Trypanozoon*	PFRA	LAMP	10
Njiru et al[36]	*Trypanozoon*	RIME	LAMP	0·001
Mugasa et al[37]	*Trypanozoon*	18S rRNA	NASBA	0·005
Mugasa et al[38]	*Trypanozoon*	18S rRNA	NASBA oligochromatography	0·005
Harris et al[39]	*Trypanozoon*	Satellite DNA and RIME	Branched DNA	10
Radwanska et al[40]	*Trypanozoon*	18S rRNA	PNA-FISH	1
Radwanska et al[41]	*T brucei gambiense*	TgsGP	Single and nested PCR	100 (single) and 1 (nested)
Mathieu-Daudé et al[42]	*T brucei gambiense*	kDNA	PCR and Southern blot	..
Radwanska et al[43]	*T brucei rhodesiense*	SRA	Single PCR	..
Welburn et al[44]	*T brucei rhodesiense*	SRA	Single PCR	..
Picozzi et al[45]	*T brucei rhodesiense*	SRA and GPI-PLC	Multiplex PCR	1–10
Li et al[46]	*T brucei* and *T equiperdum*	Maxicircles	Single PCR	20

ESAG6/7=expression-site-associated genes 6/7. FFLB=fluorescent fragment-length barcoding. *GPI-PLC*=glycosylphosphatidylinositol phospholipase C gene. ITS1=first internal transcribed spacer. kDNA=kinetoplast DNA. LAMP=loop-mediated isothermal amplification. MDA=multiple displacement amplification. NASBA=nucleic acid sequence-based amplification. *PFRA*=paraflagellar rod protein A gene. PNA-FISH=peptide nucleic acid fluorescence in-situ hybridisation. RIME=repetitive insertion mobile element. rDNA=ribosomal DNA. rRNA=ribosomal RNA. *SRA*=serum-resistance-associated gene. *TgsGP*=*Trypanosoma brucei gambiense*-specific glycoprotein gene. *One parasite contains about 0·1 pg genomic DNA. †As tested in the study by Cox et al.[29]

***Table*: Reported detection limits of different molecular diagnostics for sleeping sickness**

control programmes use a threshold of more than 5 cells per µL cerebrospinal fluid, but in some countries a threshold of more than 10 cells per µL or even more than 20 cells per µL is applied.[13–16]

Without an accurate serological screening test for *T brucei rhodesiense* infection and its acute character, the disease is generally diagnosed when patients seek medical care. The presence of parasites in the lymph, blood, and cerebrospinal fluid can be assessed by the same parasite detection tests as described for *T brucei gambiense*. Apart from primary diagnosis and staging, diagnostic tests also serve to assess treatment efficacy. As recommended by WHO, treated sleeping sickness patients should be followed up for 2 years before a decision on treatment outcome can be taken.[17] High therapeutic failure rates have been reported in several foci after treatment of the neurological phase with melarsoprol.[18] Studies on combination therapy to decrease failure rates and to improve treatment safety and feasibility are underway,[19] as well as studies on shortening the follow-up time needed to detect relapses.[20]

Because of the generally low sensitivity of the available parasite detection tests, a substantial number of infected cases are missed and not given treatment.[21,22] This not only leads to prolonged suffering and even death of patients, but also has major consequences at the community level because infected people act as reservoirs for *T brucei gambiense* sleeping sickness. Furthermore, sensitive diagnostics are not only needed for case detection and management but also for disease surveillance, monitoring of intervention studies, and epidemiological studies. In the context of the latter, diagnostics that are able to identify trypanosomes down to the subspecies level could have major added value.

Molecular parasite detection: PCR and beyond

PCR was introduced more than 20 years ago,[23] and allows production of billions of copies of a short target DNA sequence in less than 2 h by an enzymatic reaction in vitro. The technique was rapidly heralded as the ultimate tool for the detection of infectious agents because it combines sensitivity with specificity. Over the past two decades, dozens of PCRs, its derivatives, and alternative nucleic-acid-amplification techniques have been designed for the detection and speciation of trypanosomes. Since many research laboratories can

assemble their own amplification tests by designing new primers and optimising an in-house protocol, many PCRs and related techniques are currently in use for the detection and identification of the parasite. The table gives an overview of the most important molecular diagnostic techniques for sleeping sickness and their detection limits. However, note that these detection limits are simply indicative, because conditions and specimens used are not standardised or externally controlled. Comparing several molecular tests on the same specimen panel would allow more accurate comparison of the analytical sensitivities.

Subgenus, species, and subspecies specificity in PCRs

The sensitivity and specificity of a diagnostic PCR largely depends on the DNA sequence targeted by the primers. Hence, sequences that are conserved but unique for the target group and that occur as multiple copies in the parasite's genome are attractive as templates in diagnostic PCRs. In 1989, Moser and colleagues[24] developed a highly sensitive PCR that targeted the 177 bp satellite DNA within the subgenus *Trypanozoon* (*T brucei*, *Trypanosoma evansi*, and *Trypanosoma equiperdum*). The same subgenus is detectable by PCRs that amplify the expression-site-associated genes 6 and 7,[25,26] but the sensitivity is expected to be lower because both genes exist as about 20 copies,[47] whereas the parasite genome contains thousands of copies of satellite DNA.

Differentiation of the *Trypanozoon* subgenus from other *Trypanosoma* species circulating in tsetse flies or reservoir animals was reported as feasible by amplifying the internal transcribed spacer region of the ribosomal RNA gene cluster because of the inter-species length variation of this region.[27] Sensitivity was later increased by designing improved primers targeting the internal transcribed spacer and by nested PCR, a technique in which two subsequent PCRs are done.[28–30] Recently, Hamilton and colleagues[31] developed a high-throughput approach to detect inter-species ribosomal DNA sequence variability by fluorescent fragment-length barcoding.

Within *Trypanozoon*, only *T brucei gambiense* and *T brucei rhodesiense* are pathogenic for human beings and the molecular differentiation of these subspecies is mainly based on two genes. The gene encoding *T brucei gambiense*-specific glycoprotein (*TgsGP*) is only present in *T brucei gambiense*, whereas the gene encoding the serum-resistance-associated protein (*SRA*) is specific for *T brucei rhodesiense*.[48] PCRs that can discriminate both subspecies have been developed,[41–44] and are of high value for epidemiological studies, particularly given the imminent risk of overlap of both subspecies in Uganda.[49] In this context, accurate subspecies identification might become increasingly clinically important because the drugs used to treat *T brucei gambiense* and *T brucei rhodesiense* infections are different.[50]

Although the sensitivity of the *Trypanozoon*-targeting PCRs is generally sufficient for most applications, this is less the case for the subspecies-specific PCRs because both *TgsGP* and *SRA* are single-copy genes. Radwanska and colleagues[41] showed that the sensitivity of *TgsGP* PCR can be increased by doing a nested PCR using the same primers and reaction conditions. Recently, Picozzi and colleagues[45] designed a multiplex PCR that can discriminate between the *SRA* gene and variant surface glycoprotein genes with similar DNA sequences and that also indicates whether sufficient *T brucei* sensu lato DNA is present to detect a single-copy gene. Alternative *T brucei rhodesiense*-specific DNA sequences should not be expected soon because this subspecies is genetically closely related to *T brucei brucei* and the *SRA* gene might be the only molecular marker able to differentiate both subspecies. However, multicopy DNA sequences specific for *T brucei gambiense* might soon be revealed because the complete genome of this subspecies is currently being sequenced.

By contrast with the other human pathogenic kinetoplastids (*Leishmania* spp and *Trypanosoma cruzi*), the extra-nuclear minicircle kinetoplast DNA (kDNA) has not been a popular PCR target to differentiate the different members of the *Trypanozoon*. Besides an early report on the identification of *T brucei gambiense* by Southern blotting with a specific kinetoplast DNA probe,[42] no further studies were reported on molecular diagnostics that targeted the *T brucei* minicircle kDNA. Based on the absence of maxicircle kDNA in *T evansi*,[51] Li and colleagues[46] developed a PCR approach for the specific detection of *T brucei* and *T equiperdum*.

From high tech to low tech

Although most of the available diagnostic PCRs consist of an amplification step followed by amplicon electrophoresis in agarose gels, some interesting alternative approaches have emerged in recent years. Morrison and colleagues[32] showed that whole-genome amplification by multiple displacement amplification before PCR is useful if limited template DNA is available. In 2004, a real-time PCR for the detection of *Trypanozoon* was developed based on the amplification of a part of the 177 bp satellite DNA in the presence of SYBR green.[33] The investigators reported a similar analytical sensitivity to conventional PCR targeting the same satellite DNA, but the major advantages of this real-time format are its quantitative characteristics, the single-tube format, and the short reaction time. Efforts to simplify the amplicon detection step have recently been made through the development of a rapid dipstick device to detect PCR-amplified *Trypanozoon* DNA (figure 1).[34] In this oligochromatographic approach, PCR products can be detected in 5 mins and internal migration and amplification controls are incorporated in the test.

The recently developed isothermal amplification methods further simplify molecular parasite detection because they circumvent the sophisticated equipment that is needed for real-time or conventional PCR. In

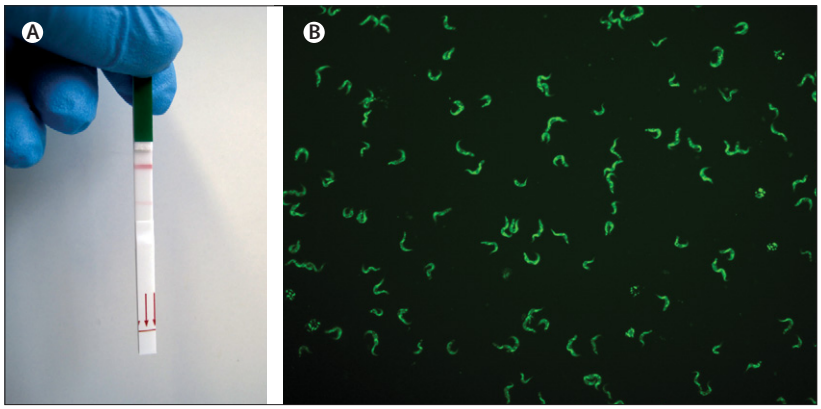

Figure 1: **Examples of trypanosome detection techniques**
Positive *Trypanozoon* OligoC-TesT dipstick result (Coris BioConcept, Gembloux, Belgium). The upper red line is the control line and the lower red line is the test line (A). Microscopic detection of *Trypanosoma brucei* parasites by fluorescence in-situ hybridisation (B).

2003, Kuboki and colleagues[35] developed two loop-mediated isothermal amplification (LAMP) reactions, one targeting the paraflagellar rod protein A of the *Trypanozoon* and a second amplifying the ribosomal P0 subunit protein of *Trypanosoma congolense*.[35] Later, Njiru and colleagues[36] reported a much more sensitive LAMP for the detection of *Trypanozoon*, in which the repetitive insertion mobile element is amplified. The investigators reported an analytical sensitivity of 0·001 parasite and the possibility to detect amplified DNA during the amplification reaction by the addition of SYBR green. However, the diagnostic accuracy of these LAMP assays still needs to be confirmed on clinical specimens from patients with sleeping sickness. LAMP is being presented as a promising technique for point-of-care diagnosis of sleeping sickness. However, one should realise that diagnosis of this disease is most often done in very remote areas devoid of even basic facilities. LAMP requires DNA extraction, electricity, a cold chain, multiple manipulations, and sufficient infrastructural measures to avoid carry-over contamination. Therefore, we are not convinced of its future as a point-of-care diagnostic test.

Whereas LAMP amplifies DNA without the need for thermal cycling conditions, a similar technique for RNA amplification has recently been introduced.[37] The *Trypanozoon*-specific real-time nucleic acid sequence-based amplification (NASBA) assay detects the parasite's 18S ribosomal RNA with an analytical sensitivity of 10 trypanosomes per mL blood. By merging this isothermal and sensitive NASBA with the simple and rapid detection by membrane oligochromatography, Mugasa and colleagues[38] further decreased the technical and infrastructural requirements needed for molecular parasite detection. However, extra caution should be taken during extraction and specimen storage because NASBA targets RNA, which is much more liable to degradation than DNA.

Although such simplifications will definitely facilitate the implementation of molecular diagnostics in several control strategies for sleeping sickness, they are still too complex for general use in first-line diagnosis and management of patients. Although they are less sensitive, amplification-independent techniques to detect the parasite's DNA or RNA are therefore a promising alternative. More than 10 years ago, Harris and co-workers[39] started exploring this domain by the development of a nonradioactive branched-DNA-based technique for direct visualisation of repetitive DNA of the parasite. However, a user-friendly format was only recently developed by combining fluorescence in-situ hybridisation (FISH) and peptide nucleic acid probes directed towards the parasite's ribosomal RNA (figure 1).[40] Nevertheless, further optimisation to increase the sensitivity and bypass the need for electricity is required before this technique can be implemented in primary diagnosis. Hence, the recent introduction of battery-powered light-emitting diode (LED)-based fluorescence microscopes, such as the Space Mike series (CytoScience, Fontaines, Switzerland),[52] Primo Star iLED (Carl Zeiss Microimaging, Göttingen, Germany), CyScope (Partec, Görlitz, Germany), or L3201-LED (Wuzhou New Found Instrument Company, Guangxi, China), could be a major breakthrough for this FISH test. Additionally, an exciting alternative approach to visualise the trypanosomes in a blood specimen is proposed by using RNA aptamers directed towards the surface coat of the parasite.[53] However, one should bear in mind that the maximum volume of blood that can be examined on a microscope slide is about 20 µL. Hence, prior concentration steps from a larger blood volume will be indispensable to reach the same analytical sensitivity as mHCT or mAECT.

Role of molecular diagnostics today

Infection with *T brucei gambiense* or *T brucei rhodesiense* is thought 100% fatal if not treated. Hence, molecular diagnostics that combine high sensitivity with specificity could play an important part in the diagnosis and staging of sleeping sickness patients and in the follow-up after treatment. However, the current molecular diagnostic formats are not yet applicable at the primary-care level. This is mainly because sleeping sickness typically affects rural populations in sub-Saharan Africa, where diagnostic laboratory facilities are basic, if they exist at all. Specialised mobile teams, hospitals, and health centres dealing with patients with sleeping sickness most often encounter infrastructural limitations and thus only apply less sophisticated diagnostic methods such as microscopic examination of thick blood film and lymph-node aspirate (figure 2). If electricity and centrifuges are available, mHCT and mAECT can be deployed in the absence of a fully functional cold chain. In this context, techniques based on enzymatic amplification of the parasite's nucleic acids are not an option for first-line diagnosis of sleeping sickness in endemic areas. FISH at ambient temperature

might be applicable in field settings if it is combined with prior parasite concentration and with a very simple fluorescence detection system.

Until now, the major role of PCR and related techniques has been confined to clinical and epidemiological research.[54–56] In such studies, samples are collected in the field and transported to central reference laboratories where they are analysed by PCR or other molecular techniques. Stabilisation of blood specimens on Flinders Technology Associates or similar filter cards (Whatman Inc, Clifton, NJ, USA) has definitely simplified transport from the field to the laboratory, but the volume of blood that can be used in DNA extraction is rather limited. Because the parasite load in blood can be very low, especially for *T brucei gambiense* infections, guanidine-based storage buffers might therefore be a good alternative.[34]

An important and appropriate point of criticism is the clinical significance of parasite DNA or RNA detection in the blood or cerebrospinal fluid of an individual. Does a positive molecular test result indicate a current infection in all cases and does every infection lead to disease? We do not yet have clear answers to these questions, and thus cannot consider molecular diagnostics as the golden bullet, as is often done. This underscores again the importance of proper test evaluation, including studies that go beyond case-control design. To our knowledge, no studies have yet been done in which PCR-based tests are validated in large-scale prospective phase 3 evaluation studies. Recently, a phase 3 study has shown that, in a visceral-leishmaniasis-endemic region, PCR provides a marker for infection rather than for clinical disease.[57] Additionally, although not yet proven, integration of *T brucei* DNA in the human genome cannot be excluded, because this occurrence has been described for *Trypanosoma cruzi*.[58] This possibly might explain the contradictory results by PCR and antibody detection tests.[59–61]

Several studies have been reported in which PCR methods were used to reveal atypical human infections caused by *T brucei brucei*,[62] *T evansi*,[63] *T congolense*,[64] and *Trypanosoma lewisi*.[65] Although the practical use of molecular identification of trypanosomes might be limited to such occasional research questions, these reports clearly show the power of these methods. However, because such PCR-based approaches are able to identify trypanosomes down to the subspecies or even strain level,[41–46,66] they are very useful in studies on reservoir identification and control,[28,67,68] and disease epidemiology.[69,70]

Furthermore, molecular parasite detection and identification has potential for veterinary use. Animal trypanosomiasis is responsible for severe losses in the agricultural sector worldwide and the accuracy of current diagnostic tests is poor. Farmers and veterinary personnel often treat sick animals and use the response to treatment as a retrospective diagnosis. However, the presence of concomitant diseases may mask trypanosomiasis. As for sleeping sickness, molecular diagnostics are probably no option yet for primary diagnosis of animal trypanosomiasis, but might be relevant for disease surveillance and monitoring of control programmes.

Figure 2: **Microscopic diagnosis of sleeping sickness in a village near Mbuji-Mayi, DR Congo**

Conclusions and perspectives

Although a wide range of sensitive molecular tests for parasite detection and differentiation are available for research laboratories and reference centres with molecular biology facilities, the direct benefit for the patient remains rather limited. None of the current molecular diagnostics can be implemented in routine diagnosis of sleeping sickness at the primary health-care level. According to WHO's global plan to combat neglected tropical diseases,[71] simple and safe diagnostic tools should be developed that can be integrated into health systems in resource-limited settings. Hence, targeted efforts are needed to develop molecular diagnostics that are sensitive and specific, simple to perform and to interpret, do not require expensive or elaborated equipment, and are thermostable for prolonged storage. Last, but not least, they should be cost effective. The price of a diagnostic test has to be outlined in the global cost of patient care. It might be appropriate to invest in more expensive but better diagnostics if this can reduce the cost of patient care, particularly given the long and costly treatment schedules for sleeping sickness and the even longer follow-up after treatment.

Besides the need for formats that are applicable in field conditions, standardisation and proper test evaluation are of utmost importance for successful integration of molecular diagnostics in disease control programmes. After its development, each new test should be assessed in subsequent phase 1, 2, and 3 studies followed by specific studies to show the test's use and effect.[72] Very few evaluations of molecular tests went beyond phase 1 because most have just been assessed on a small number

of samples in the same laboratory in which they were developed. The real diagnostic value of a new test can only be measured when evaluated in settings in which it will be applied in the future. All too often, investigators label a new molecular test as "field applicable", but we did not find any reports of phase 3 evaluations of molecular diagnostics in such settings. By targeting simplification, standardisation, and proper test evaluation, we might be able to move the powerful molecular platform from the research institutes to the field.

Contributors

SD searched the published work and wrote the first draft of the paper. PB provided critical comments on the content and reviewed the subsequent drafts. Both authors approved the final version.

Conflicts of interest

We declare that we do not have any conflicts of interest.

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