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ORIGINAL PAPER

Antibodies to a novel antigen in acute hepatitis C virus infections

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Background Conformational viral proteins potentially play an important role in the immunobiology of acute hepatitis C virus (HCV) infection and may enable earlier antibody detection.

Materials and Methods HCV RNA was detected using nucleic acid testing. Early antibody production was evaluated using three enzyme immunoassays (EIAs) containing antigenic proteins not present in licensed EIAs. Respectively, these contained:

(1) multiple-epitope fusion antigen (MEFA) 7·1-NS3/4a,
(2) F and Core, and
(3) E1/E2 proteins.

NS3/4a is a conformational antigen retaining protease and helicase enzymatic activities. MEFA 7·1 contains the linear epitopes used in licenced EIAs, including the latest EIA-3·0, in combination with genotype 1–3 specific epitopes. Forty-two RNA positive, EIA-3·0 negative samples, including two persistently serosilent cases, were used to evaluate these research EIAs. As controls, 54 EIA-3·0 negative/RNA negative and three HCV RNA+/antibody positive specimens were included.

Results Only the MEFA 7·1-NS3/4a EIA was positive in seven (17%) of the 42 HCV RNA + specimens, in all three EIA-3·0 positive controls but in none of 54 EIA-3·0 negative/HCV RNA negative controls. Notably, six of the seven (86%) specimens had evidence of active hepatitis (ALT > 210 IU/l). The two serosilent cases were research EIA negative.

Conclusion A novel EIA with conformational and linear epitopes detected HCV antibodies in 17% of viraemic specimens missed by the standard reference EIA-3·0. Our research EIA appears to detect HCV antibodies closer to the initiation of acute hepatitis. Given that the average RNA-positive, antibody-negative window period is ~56·4 days, this 17% yield would translate into a ~10-day earlier detection of antibodies.

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Introduction

Hepatitis C virus (HCV) is a prevalent pathogenic blood-borne infection with an estimated 170 million persons infected worldwide [1,2]. Commercially licenced HCV enzyme immunoassays (EIA), which are deployed to screen for HCV exposure, use recombinant proteins presenting linear epitopes. The specificity and sensitivity of these linear epitope-based assays has been extensively discussed [3–5]. Recognized however, is that conformational antigens may play an important role in the very early stages of immunological engagement in HCV-infected individuals [6]. Nevertheless, the immunogenic impact of conformational HCV proteins in early infection is not well understood.

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The HCV genome is a ~9·6-kb positive-strand RNA from which a single polyprotein of between 3010 and 3033 amino acids is expressed. This polyprotein is cleaved by cellular and viral proteases to yield both structural (a nucleocapsid or 'core', two envelope glycoproteins and P7) and non-structural (NS2 to NS5) proteins [7]. The NS3 and NS4A regions contain viral protease and helicase activity [7]. The HCV 3·0 EIA (Ortho Clinical Diagnostics, Raritan, NJ) includes recombinant proteins representing linear epitopes from the core, NS3, NS4 and NS5 regions [8] and is used to screen approximately 80% of the blood supply in the USA for antibodies to HCV.

In 1999, based on advances in diagnostic testing, a new donor-screening methodology, nucleic acid amplification testing (NAT), was implemented in the USA under a Food and Drug Administration (FDA)-approved investigational new drug protocol [9–11]. This allowed for detection of HCV RNA positive, but antibody non-reactive, infected units of blood during routine blood-donor screening. Very rarely, some HCV-infected people apparently fail to ever produce specific antibodies, but most samples of this type (RNA positive/antibody negative) simply represent very early HCV infection. Such donations provide opportunity for evaluating earlier HCV antibody detection systems and for investigating the B cell immunobiology of early HCV infection. We therefore tested three research EIAs, containing E1/E2, F/Core and multiple-epitope fusion antigen (MEFA) 7·1-NS3/4a proteins which are not present in commercially available assays. The rationale behind using these three research EIAs was the concept of delayed antibody detection by HCV 3·0 EIA due to either absence of the antigen (F protein [12] and E1/E2 [13,14] antigens) or new and uniquely configured antigens (MEFA 7·1 and NS3/4a [15]) with heightened sensitivity to non-genotype 1 specimens. To investigate the presence of previously undetected anti-HCV antibodies, we evaluated 42 'NAT yield' specimens, defined as HCV RNA positive but HCV antibody negative using EIA 3·0.

Materials and Methods

Study population

Forty-two cross-sectional NAT yield specimens identified during blood donor screening at the American Red Cross from March 1999 through January 2002 were evaluated. A NAT yield sample was defined as an allogeneic donation that was

(1) non-reactive for HCV antibody using HCV 3·0 EIA (Ortho-Clinical Diagnostics);

(2) RNA positive by NAT screening (Procleix Assay, Gen-Probe, San Diego, and Chiron, Emeryville, CA); and

(3) was confirmed positive for HCV RNA using an alternative NAT assay (SuperQuant[16], National Genetics Institute, Los Angeles, CA) [17].

Among the 42 cross-sectional NAT yield specimens, there were two donors (5%) who remained viraemic with normal alanine aminotransferase (ALT) levels, but failed to seroconvert after ~340 days and ~595 days of follow-up, respectively [18,19]. These are referred to as 'serosilent' cases.

As controls, 54 false-positive NAT specimens were evaluated. A false-positive specimen was one detected by initial NAT screening, but unconfirmed by parallel and subsequent RNA and HCV 3·0 EIA testing. The controls also included three HCV RNA positive, HCV 3·0 EIA positive specimens.

Research EIA testing was performed at Chiron Corporation on encrypted NAT yield and controls specimens. Chiron's results on these encrypted specimens were interpreted at Blood Systems Research Institute.

Routine donor screening assays

All specimens were routinely tested by HCV 3·0 EIA, RIBA HCV 3·0 Strip Immunoblot Assay (Chiron Corporation), alamine aminotransferase (ALT in IU/l), and transcription-mediated amplification using the Procleix Assay [17]. If the donors of the HCV NAT yield specimens used in this study agreed, they participated in a standardized prospective protocol used to enroll them into follow-up with specimens collected at approximately 4-week intervals through HCV 3·0 EIA seroconversion and beyond [17].

Viral load testing

Viral load testing was performed using the SuperQuant assay [16]. In this assay, purified viral RNA is subjected to reverse transcription to make cDNA copies. The resultant cDNA undergoes four separate polymerase chain reactions (PCR) at 25, 30, 35, and 45 cycles using two specific primer sets from the 5-UTR of the HCV genome. The use of four separate reactions increase the linear range of quantification (i.e. 100 copies/ml to 5 000 000 copies/ml).

Research enzyme immunoassay

All NAT yield and control specimens were evaluated by three research EIAs. In all three research EIAs the cut-off was determined using plasma from a 1000 screening negative random blood donors. The mean optical density from testing these 1000 donor samples plus seven standard deviations was used as the cut-off for each EIA.

The first EIA contained two antigenic proteins: a conformational antigen that retains both protease and helicase enzymatic activities (NS3/4a), and a single multiple-epitope fusion protein (MEFA 7·1). MEFA 7·1 represents an enhanced antigen incorporating known major epitopes of the HCV polyprotein [15]. Expression of MEFA 7·1 in

yeast produces proteins from the HCV Core, E1, E2, NS3, NS4, and NS5 regions with genotypes 1–3 specific epitopes for subtype-specific regions of the core, E2 and NS4 proteins. In previous experiments, this EIA demonstrated stronger immunoreactivity to all HCV genotypes as well as detecting seroconversion earlier than HCV 3·0 EIA [15].

The second research EIA used the F and core proteins of hepatitis C as the anti-HCV detection antigens [12,20]. The F ('frameshift') protein is an HCV gene product that is expressed by a ribosomal frameshift within the capsid-encoding sequence [12]. The F protein was coated onto Costar High Binding plates at 100 ng/well using Ortho HCV 3·0 Coating buffer. Plates were blocked using phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) and stabilized using PBS/BSA/3% sucrose before drying. For the assay, 20 µl of donor specimen was diluted in 200 µl of Ortho HCV 3·0 sample diluent and added to the assay plates coated with F and core proteins. The samples were incubated for 1 h at 37 °C, and then washed. The hydrogen peroxide (HRP) conjugate and substrate o-phenylenediamine (OPD) were added as described previously [15].

The third research EIA used an E1/E2 protein generated in Chinese Hamster Ovarian (CHO) cells as the anti-HCV detection antigen [20,21]. This antigen is able to detect specific antibodies in seroconversion panels as early as both the core and NS3 antigens, and is capable of detecting both conformational and linear antibody epitopes [14,22]. The plates were coated with 100 ng/well of purified E1/E2, and the assay was performed similarly to the F and core protein assay as described above.

Statistical analysis

Significance calculations were performed using STATVIEW 5·0·1 (SAS Institute Inc). The estimates for mean time from index donation to HCV 3·0 EIA seroconversion used interval censored methodology.

Results

As seen in Table 1, among the 42 NAT yield specimens, seven (17%) specimens were reactive in the research EIA containing the NS3/4a and MEFA 7·1 antigens; while 35 (83%) specimens were non-reactive, including the two specimens from the persistently serosilent cases. Conversely, all 42 NAT yield specimens, including the two serosilent cases, were non-reactive in the research EIA assays using both the F/core protein combination and the CHO E1/E2 antigens. All 54 NAT false-positive, HCV 3·0 EIA non-reactive control specimens (100%) were non-reactive when evaluated by all three research EIAs. Either two out of three or all three RNA positive, HCV 3·0 EIA positive controls were reactive in the research assays containing the MEFA 7·1-NS3/4a, Core/F proteins, and the E1/E2 antigens (Table 1). Table 2 summarizes the measured parameters in all seven NAT yield specimens in which HCV antibodies were demonstrated using the MEFA 7·1-NS3/4a EIA. Among the 42 NAT yield specimens there were 10 (24%) with an ALT value > 100 IU/ml (Fig. 1). Six of these 10 NAT yield specimens (60%) were reactive in the MEFA 7·1- NS3/4a research EIA. Consequently, six (86%) of the seven NAT yield specimens reactive in the MEFA 7·1-NS3/4a research EIA had evidence of significant hepatitis represented by ALT values > 100 IU/ml; while only four of the 35 (11%) MEFA 7·1-NS3/4a research EIA non-reactive specimens had ALT values > 100 IU/ml (Fig. 1). This difference is highly significant ($P < 0{\cdot}00001$) and implies that the MEFA 7·1-NS3/4a research EIA detects antibodies synthesized during early active hepatitis (ALT 210–1433 IU/l). Four of the seven (57%) NAT yield specimens reactive in the MEFA 7·1-NS3/4a research EIA were followed to HCV 3·0 EIA seroconversion (Fig. 2a); while 17 of the 35 (49%) MEFA 7·1-NS3/4a non-reactive NAT yield specimens were followed to HCV 3·0 EIA seroconversion (Fig. 2b). Using interval-censored methodology, the mean time from index to HCV 3·0 EIA seroconversion among the four MEFA 7·1-NS3/4a reactive specimens was 12 days [95% confidence interval (CI) 1, 36 days]; while

Table 1 Research EIA performance on NAT yield and control specimens

		Controls	
	NAT yield specimens (HCV RNA positive HCV 3·0 negative)	**HCV RNA negative HCV 3·0 EIA negative**	**HCV RNA positive HCV 3·0 EIA positive**
Number of samples	42	54	3
Detected by first research EIA (MEFA 7·1-NS3/4a)	7 (17%)	0	3 (100%)
Detected by second research EIA (F and Core Proteins)	0	0	2 (67%)
Detected by third research EIA (CHO E1/E2)	0	0	3 (100%)

Table 2 Distribution of measured parameters for MEFA 7·1-NS3/4a EIA reactive donors

Specimen ID no.	Donor type[a]	Sex	EIA 3·0 S/CO[b]	Res. 1 S/CO (MEFA 7·1-NS3/4a)	Res. 2 S/CO (F/core)	Res. 3 S/CO (E1/E2)	ALT	Viral load (cps/ml)	Genotype
1	FT	F	0·046	5·2	0·015	0·000	28	7700	1a/1b
2	FT	M	0·01	5·0	0·008	0·376	135	7 200 000	3a
3	RPT	M	0·013	1·1	0·08	0·031	210	75 000	2b
4	RPT	F	0·426	3·2	0·016	0·004	229	19 000 000	1a
5	RPT	M	0·016	2·2	0·010	0·083	422	51 000 000	1a
6	RPT	M	0·033	1·3	0·011	0·363	687	25 000 000	1a
7	FT	F	0·61	3·7	0·028	0·002	697	36 000 000	3a

[a]FT, first time donor; RPT, repeat donor. [b]S/CO, signal-to-cut-off ratio.

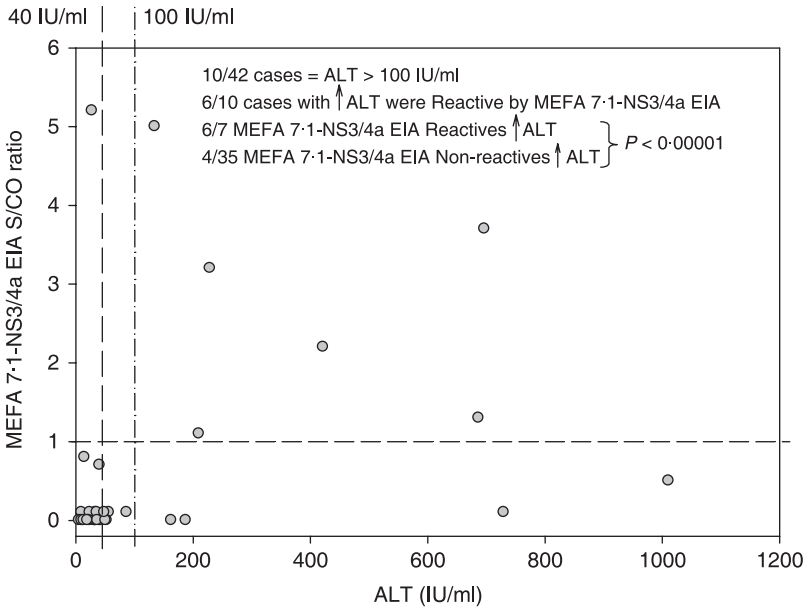

Fig. 1 The *y*-axis represents MEFA 7·1-NS3/4a results as a signal-to-cut-off (S/CO) ratio. MEFA 7·1-NS3/4a EIA positive reactivity was defined as an S/CO ratio equal to or greater than 1·0. A S/CO ratio < 1·0 in the MEFA 7·1-NS3/4a EIA was considered non-reactive. All MEFA 7·1-NS3/4a EIA reactive and non-reactive NAT yield specimens are depicted by (○). The *x*-axis represents ALT values in IU/ml.

among the 17 MEFA 7·1-NS3/4a non-reactive specimens it was 41 days (95% CI 27, 54 days) (Fig. 2b). The window-period reduction of the MEFA 7·1-NS3/4a research EIA was calculated using the following formula and assumptions: $7/42 \times 56{\cdot}3$ days = 9·4 days [23]. The numerator (7) represents the number of MEFA 7·1-NS3/4a research EIA reactive specimens, while the denominator (42) is the total number of NAT yield specimens evaluated and 56·3 is an estimated average in days [23] of the viraemic plateau phase preceding seroconversion. The 95% CI for this estimate is (3·0, 15·8) days. The 9·4 day advanced detection using MEFA7·1-NS3/4a is consistent with previous observations [15]. No correlation between reactivity in the MEFA 7·1-NS3/4a research EIA and viral load was observed (data not shown). The viral loads for the specimens that were non-reactive in the MEFA 7·1-NS3/4a research EIA ranged from 10^2 to $9{\cdot}3 \times 10^7$ copies/ml, while the viral loads for the reactive specimens ranged from $7{\cdot}7 \times 10^3$ to $5{\cdot}1 \times 10^7$ copies/ml.

As previously described, among the 42 cross-sectional NAT yield specimens, there were two donors (5%) who remained viraemic, but failed to seroconvert by HCV 3·0 EIA after ~340 days and ~595 days of follow-up, respectively. No antibodies to HCV were detected in these two persistently serosilent donors by any of the three research EIAs used in this study. The HCV genotype of the first donor was 3a while the genotype of the second donor was 2b. The respective viral loads ranged from $3{\cdot}3 \times 10^5$ to $1{\cdot}4 \times 10^7$ copies/ml and from $2{\cdot}9 \times 10^5$ to $2{\cdot}8 \times 10^7$ copies/ml. During the periods of observation the viral loads fluctuated but never became undetectable.

Discussion

Our results indicate that antibodies with conformational determinants are present in at least some people – 17% shown here – during early HCV infection, prior to antibody detection

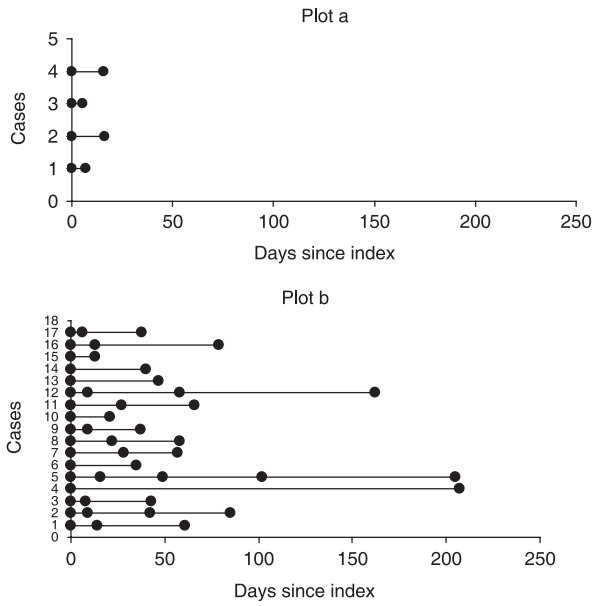

Fig. 2 Plot (a) represents the MEFA 7·1-NS3/4a reactive specimens (n = 4); while plot B represents the MEFA 7·1-NS3/4a non-reactive specimens (n = 17). 'Time 0' for each panel was defined as the 'NAT Yield specimen' when HCV RNA was detected by transcription-mediated amplification. Only the NAT-yield specimens were evaluated by the MEFA 7·1-NS3/4a assay. The last time point for each case represents the first follow-up specimen at which HCV 3·0 EIA seroconversion was detected. The median time from HCV RNA detection to HCV 3·0 EIA seroconversion for the 4 MEFA 7·1-NS3/4a reactive specimens in Plot (a) was 12 days (95% CI 1, 36); while the same calculation among the 17 MEFA 7·1-NS3/4a non-reactive specimens in Plot (b) was 41 days (95% CI 27, 54).

using the latest FDA-licensed HCV 3·0 EIA. Previous experiments using the MEFA 7·1-NS3/4a assay and anti-core protein seroconversion panels support the inference that our differential antibody detection is primarily due to the NS3/4a conformational antigen [15]. Notably, the E1/E2 antigens, which are also conformational and were correctly glycosylated having been expressed in CHO cells [14], were unable to detect HCV-specific antibodies in advance of EIA-3·0. The E1/E2 antigens are ostensibly more readily accessible for antibody binding at the external surface of the HCV virion. This suggests intriguing and potentially immunologically relevant differences between structural and non-structural HCV proteins during early infection.

Our observations trigger broader questions about the properties and relevance of antibodies detected using commercially available vs. research HCV EIA assays. Denatured linear epitopes may identify antibodies that exhibit different biological effects from conformational antibodies. Potential differences in affinity, viral selection pressure and disease outcome between the different conformational antibody subsets generated during early HCV infection will require further investigation. Notably, among the 42 NAT yield specimens included in this study, six of the 10 (60%) cases with enzyme elevation, indicating active hepatitis, were reactive in the MEFA 7·1-NS3/4a research EIA, and six of the seven (86%) MEFA 7·1-NS3/4a reactive cases had active hepatitis (ALT 210–1433 IU/l). This distribution is highly significant ($P < 0{\cdot}00001$) and suggests that the MEFA 7·1-NS3/4a research EIA is able to detect humoral immunity concordant with hepatocyte damage, perhaps reflecting the strength of the overall HCV-specific immune response and early presentation to B cells of non-structural HCV proteins [24,25].

Reported instances of persistent HCV RNA positive, antibody non-reactive blood donors are exceptional (i.e. one out of 7·7 million blood donations [17,18] and one out of 2 million blood donor plasma units [26]). Two such donors (5%) included in our study remained viraemic with normal ALT levels and failed to seroconvert after ~340 days and ~595 days of follow-up, respectively [17,18]. Subsequent to this study these two donors were followed for a total of 906 and 1782 days (i.e. ~2·5 and ~4·88 years) and remained HCV RNA positive, HCV 3·0 EIA non-reactive. Failure of these two cases to develop an antibody response to the linear and conformational antigens present in all of our EIA assays seems to indicate a broad inability to initiate a humoral immune response despite large amounts of antigen, i.e. high HCV viral loads. We are not yet aware if this apparent anergy extends to the T-cell compartment or if it has clinical correlates in terms of disease progression or of its underlying mechanism.

Until recently, most of what was known about the kinetics of viraemia, ALT elevation, and seroconversion in very early phase HCV infection was based on cases of infection in transfusion recipients or in experimentally infected chimpanzees [23]. Based on observations from these two populations, the early dynamics of HCV infection are comprised of four phases. HCV RNA is detectable within 2–14 days of exposure and followed by the first phase termed the 'ramp-up phase'. During this phase the viral load increases exponentially [23]. The second phase is termed the 'plateau phase'. This phase occurs between ramp-up and seroconversion and is estimated to last 56·3 (95% CI, 44·8–67·8) days [23]. During this phase high viral loads are maintained. The third phase, ALT elevation, generally occurs after 6–112 days (median, 46 days) after exposure [27]. The fourth and last phase is seroconversion. HCV specific antibodies are detectable within 20–150 days (mean length of time, 60 days post-transfusion or infection) [23,28]. If our observations are incorporated into this model, then the sequence of events would be: (1) viral load ramp-up; (2) viral load plateau; (3) ALT elevation; (4) MEFA 7·1-NS3/4a research EIA seroconversion; and finally (5) HCV 3·0 EIA seroconversion. In this model there would be an overlap between MEFA 7·1-NS3/4a research EIA seroconversion and ALT elevation. A study using sequential specimens from 21 post-transfusion HCV infections [29] indicated a mean length of 16·7 days between the first elevation in ALT and detection

of anti-HCV antibody by HCV 3·0 EIA [30]. Our observations indicated that the MEFA 7·1-NS3/4a assay detects seroconversion 2·7–15·1 days earlier than HCV 3·0. Therefore, one of the effects of our observations is a shortening of the elevated-ALT preseroconversion window placing the initial synthesis of antibodies earlier than previously observed.

One final point is the disposition of the MEFA 7·1-NS3/4a research EIA. Because of current screening for HIV and HCV RNA using NAT in combination with anti-HCV screening, it is very improbable that the MEFA 7·1-NS3/4a research EIA will ever be commercialized for donor screening in either the USA, Europe or other developed countries. In the presence of NAT screening there is no real benefit of an HCV EIA with enhanced sensitivity over the HCV 3·0 EIA.

NAT screening entails substantial operational complexity as well as a sizeable capital investment, and these considerations preclude its current implementation in many developing countries [31]. On the other hand, in developing countries that screen using HCV antibody detection alone, implementation of this assay could improve the detection rate during the early stages of infection. In countries with endemic HCV infections, this could significantly reduce blood-borne transmission and prove useful for both blood donor screening and patient testing [32–34]. The performance characteristics of HCV antigen assays for the diagnosis of early HCV infection have been previously described [35]. Window period closure for the ORTHO trak-C assay was delayed either by 3·2–4·3 days or by 2–8 days depending on the study [35,36]. Alternatively, the seroconversion sensitivity of the MEFA 7·1-NS3/4a research EIA was 2 to 12 days ahead of the currently licensed Ortho HCV 3·0 assay and the Abbott PRISM in nine out of nine seroconversion panels because of its ability to detect window period associated with early ALT elevation [15]. The presence of a combination HCV antigen-antibody assay might result in enhanced blood safety.

In conclusion a novel research EIA using conformational and enhanced linear epitopes (NS3/4a and MEFA 7·1 antigens, respectively) detected HCV antibodies in 17% of viraemic donations missed by HCV 3·0 EIA. A correlation between elevated ALT levels and detection by the MEFA 7·1-NS3/4a assay raises intriguing questions about the potential clinical correlates of our observations. Future studies will address these possible implications and uses.

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