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Measuring BDNF in saliva using commercial ELISA: Results from a small pilot study



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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a protein often studied in psychiatric populations. Commercial ELISA kits have been validated for measuring BDNF in blood plasma and serum, but blood collection is an invasive method which cannot always be used. The aim of this pilot study was to explore the noninvasive alternative of measuring BDNF in saliva. Three different commercial ELISA kits were used to analyze parallel plasma and saliva samples from six healthy adults. In total 33 plasma and 33 saliva samples were analyzed according to manufacturers' standard protocols. BDNF was successfully measured in plasma in two of the three kits, of which the results correlated highly ($r_s = .88$). BDNF could not be measured reliably in saliva. The results of this pilot study suggest that techniques of commercial BDNF kits may not be ready for noninvasive saliva measurements, which limits the sampling frequency and settings.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a protein responsible for synaptic plasticity. Differences in BDNF levels have been associated with several psychiatric disorders, such as Major Depressive Disorder, bipolar disorder and schizophrenia (Autry and Monteggia, 2012; Munkholm et al., 2016; Polyakova et al., 2015; Soares et al., 2016; Toll and Mané Santacana, 2015; Wang et al., 2016). In these studies BDNF levels were determined in blood serum or plasma. Blood collection from patients or participants is an invasive method, and poses restrictions to the frequency of sampling and the environments in which sampling can take place. The background of this pilot study is a project in which anhedonia was investigated in young adults and interventions were developed to help them regain pleasure (Roekel et al., 2016). Because of its associations with depression, BDNF was considered a relevant protein. Since one of the interventions took place in a situation in which blood collection was not possible, the possibilities of the noninvasive and cost-efficient alternative of measuring BDNF in saliva were explored in a small pilot study. Although not to be interpreted as conclusive evidence, other researchers interested in measuring BDNF in saliva may benefit from the results of this pilot study. Noninvasive alternatives to blood BDNF would be relevant for, among others, research on BDNF fluctuations in psychiatric patient groups, and in the upcoming field of ecological momentary assessment (EMA), which involves frequent assessment of emotions, behaviors and

social contexts over time in one's naturalistic environment (Shiffman et al., 2008), and has already been extended to salivary biomarkers cortisol and alpha-amylase (Bitsika et al., 2015; Booij et al., 2016).

It has been demonstrated by means of immunoblotting that BDNF is present in human saliva (Mandel et al., 2009). However, whereas several commercial ELISA kits have been validated for measuring BDNF in plasma and serum, up until now none has been validated for measuring BDNF in saliva. In 2011 Mandel and colleagues assessed the possibilities of quantifying BDNF in saliva using commercial ELISA kits (Chemicon and Promega), but the BDNF levels rarely reached the minimum detection level of the kits (Mandel et al., 2011). Mandel and colleagues suggested that this was most likely due to matrix complexity, and decided not to use the commercial kits, but to develop a sandwich ELISA optimized for measuring BDNF in saliva themselves. In recent years, six studies reported to have successfully quantified BDNF in saliva by using commercial ELISA kits (de Souza et al., 2014, 2012; Ikai et al., 2014; Matsuki et al., 2014; Saruta et al., 2012; Tirassa et al., 2012). However, even reported BDNF levels for healthy young adults (Matsuki et al., 2014; Saruta et al., 2012; Tirassa et al., 2012) were very diverse, mean levels ranging from 9 pg/mL to 400 pg/mL. The studies used different ELISA kits and different procedures for collecting, storing and processing; and in most studies BDNF levels below the minimum detection threshold were interpreted as true results rather than discarded as unreliable results. See Table 1 for a comparison of the ELISA kits and procedures used in the six previous studies, as well as of

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Table 1
A comparison between studies in which successful quantification of BDNF in saliva using commercial ELISA kits was reported.

| Study | Sample | Saliva collection | ELISA kit & procedure / duplicate? | Centrifugation and freezing procedure | Dilution / Acidification | Mean levels | Detection range | Results outside detection range |
|------------------------|---|--|--|--|--|---|---|---|
| de Souza et al. (2012) | 26 adults with Burning Mouth Syndrome (BMS): 25 females; aged 63.8 ± 12 | (1) Mouth washed with filtered water (2) Saliva formed in mouth during 5 min of stimulation spitted in tube | R & D Duoset Not in duplicate | Centrifugation at 3000 rpm for 15 min Diluted and then stored at -20 °C | 1:1 dilution Acidification: phosphate-buffered saline solution containing protease inhibitors | Before therapy: ± 20 pg/mg After therapy: ± 17 pg/mg | No minimum detection level reported; according to manufacturer: 23.40 pg/mL | Of the 52 reported results, 30 are very close to 0 and 43 are below 23.40 Results outside detection range not disqualified |
| de Souza et al. (2014) | 30 adults with BMS: 29 females; aged 62.1 ± 12.7 32 controls: 31 females; aged 61.6 ± 12.8 | (112) Mouth washed with filtered water (113) Saliva formed during 5 min of stimulation spitted in tube | R & D Duoset Not in duplicate | Centrifugation at 1509 × g for 15 min Diluted and then stored at -80 °C | 1:1 dilution Acidification: phosphate-buffered saline solution containing protease inhibitors | BMS: 5.27 pg/mg, range: 0–72.14 Controls: 10.73 pg/mg, range: 0–129.20 | Study reports a minimum detection level of 10 pg/mL; according to manufacturer: 23.40 pg/mL | Means and ranges suggest many results below minimum detection level Results outside detection range not disqualified |
| Ikai et al. (2014) | 50 schizophrenia patients, 25 yoga (16% males, aged 53.5 ± 9.9) and 25 to control group (17% males, aged 48.2 ± 12.3) | Unclear how saliva was collected | Millipore CYT306, according to manufacturer's instructions | Not reported | No dilution or acidification | Yoga group: mean = 2.16 pg/mL ± 3.84 pg/mL Control group: 4.12 pg/mL ± 8.26 pg/mL | No minimum detection level reported; according to manufacturer: 15 pg/mL | Reported means are far below the minimum Results outside detection range not disqualified |
| Tirassa et al. (2012) | 16 young healthy non-smoking female students in first week of their post-menstrual period (aged 21 ± 0.5) | Samples collected at 8.00, 13.00 and 20.00 h, after fasting Passive drool in plastic tube | R & D Quantikine Human BDNF immunoassay according to manufacturer's instructions In duplicate | Centrifugation at 10,000 rpm for 10 min Stored at -70 °C | No dilution or acidification | Baseline levels: 8.00: ± 400 pg/mL 13.00: ± 360 pg/mL 20.00: ± 210 pg/mL Post-light therapy: 8.00: ± 175 pg/mL | No minimum detection level reported; according to manufacturer: 62.5 pg/mL | Unclear, since raw results for post-therapy were not reported |

(continued on next page)

Table 1 (continued)

| Study | Sample | Saliva collection | ELISA kit & procedure / duplicate? | Centrifugation and freezing procedure | Dilution / Acidification | Mean levels | Detection range | Results outside detection range |
|-----------------------|---|--|---|--|------------------------------|--|---|--|
| Matsuki et al. (2014) | 40 healthy fertile female dental hygienist students: in follicular phase (n = 24), aged 21.0 ± 3.5, and in luteal phase (n = 16), aged 23.0 ± 3.9 | Samples collected by salivettes between 1 and 4 pm Fasted 5 min prior to sampling | Millipore CYT306 according to manufacturer's instructions In duplicate | Centrifuged at 626 × g for 15 min at 4 °C and stored at -80 °C. Upon thawing, samples were centrifuged once more | No dilution or acidification | 13.00: ± 180 pg/mL 20.00: ± 170 pg/mL | A minimum detection level of < 4 pg/mL is reported; according to manufacturer: 15 pg/mL. | Many results below 15 pg/mL were reported, even below 4 pg/mL. Results outside detection range not disqualified |
| Saruta et al. (2012) | 50 healthy, non-medicated, non-smoking volunteers (26 males), aged 27 ± 6.4 | Samples were collected by means of salivettes between 9 and 10 am Fasted 2 h before sampling, no alcohol 24 h prior | Millipore CYT306 according to manufacturer's instructions In duplicate | Centrifuged at 2000 rpm for 15 min at 4 °C and stored at -80 °C. Upon thawing, samples were centrifuged once more | No dilution or acidification | Males: 40.76 ± 4.83 pg/mL Females: 52.64 ± 8.42 pg/mL | A minimum detection level of < 4 pg/mL is reported, but this does not match the manufacturer's information of 15 pg/mL. | Unclear, since raw results or ranges were not reported |

the mean BDNF levels that were reported. It thus remains unclear whether BDNF in saliva can be quantified reliably by using commercial ELISA kits.

In our study, we aimed to compare salivary BDNF with BDNF in plasma. As opposed to BDNF in serum, which has been reported not to be associated with salivary BDNF (Mandel et al., 2011; Tirassa et al., 2012), correlations between salivary BDNF and BDNF in plasma have not been studied in humans to date. Plasma BDNF levels reflect the momentary circulation of BDNF. Serum BDNF is determined by the sum of plasma BDNF and BDNF released from the platelets in serum (Montag, 2014; Polyakova et al., 2015), which have a life-span of about ten days and can accumulate or store BDNF (Dale, 1997). Therefore, serum BDNF is likely to reflect relatively stable or long-term BDNF levels, and plasma BDNF more momentary or short-term BDNF levels (Bus, 2014; Montag, 2014; Polyakova et al., 2015). Experiments in rats have shown that in situations of acute stress BDNF in the submandibular salivary gland may affect plasma BDNF levels (Tsukinoki et al., 2007).

Developing own methods for measuring BDNF in saliva, as Mandel and colleagues did, is not feasible for most researchers. Salivary BDNF would be accessible for many more researchers if commercial kits could be used for quantification. Because of the inconsistent previous results and ongoing technical developments during the past years, we conducted a pilot study in which three different commercial ELISA kits were used to measure BDNF in plasma and saliva. Main aims of the study were to explore the feasibility of quantifying BDNF in saliva with commercial ELISA kits when following the manufacturers' standard protocols, a comparison between the different ELISA kits, and a comparison between plasma and saliva levels.

2. Materials and methods

2.1. Subjects

From six participants, one male (age 43, non-smoking) and five females (age 63, non-smoking; age 28, smoking; age 51, non-smoking; age 37, non-smoking; and age 36, non-smoking), blood and saliva were collected. All participants were healthy and free of medication. From the first three participants (study 1), blood and saliva were collected in the morning on five consecutive days, resulting in a total of five blood and five saliva samples per participant. From the other three participants (study 2) blood and saliva were collected three times a day on two different days, with one resting day in between, resulting in a total of six blood and six saliva samples per participant. Participants were treated in accordance with the Declaration of Helsinki, and written consent was acquired from all participants.

2.2. Fasting procedures

There is evidence that eating and drinking prior to sample collection increases BDNF levels in serum (Bus et al., 2011) and it is generally recommended to avoid eating, drinking and tooth brushing prior to collection of saliva samples (Salimetrics LLC Company, 2012; Wong, 2009). Therefore, it would be best for participants to adhere to a fasting protocol prior to saliva and plasma collection. However, fasting is not possible in all circumstances, particularly if multiple samples per day are collected, and recommended fasting periods differ. We therefore used feasible fasting procedures, adapted to the time of the day, and around noon we tested two different conditions, because around noon it may be most difficult to adhere to a fasting protocol. In study 1, blood and saliva samples were collected after overnight fasting (no eating, drinking or brushing teeth). In study 2, morning blood and saliva samples were collected after overnight fasting and the late afternoon samples after two hours of fasting. On the first day the sample around noon was collected after two hours of fasting, on the second day after 30 min of fasting.

2.3. Blood collection

Study 1. Blood collection took place at five consecutive days, in the morning at home within half an hour after waking up, using standard sterile techniques. Blood was collected by venipuncture in 10 mL EDTA tubes (BD Biosciences, Franklin Lakes, NJ, USA). Samples were stored in a cooling bag ($\sim 8^{\circ}\text{C}$) immediately after collection, transferred to the laboratory, centrifuged at $1650 \times g$ at 4°C for 10 min. Plasma samples were aliquoted and stored at -80°C within one hour after collection.

Study 2. Blood collection took place at the University Medical Center Groningen, three times a day on two different working days: in the morning around 8:30, around noon and in the afternoon around 16:15, using standard sterile techniques. Blood was collected by venipuncture in 4 mL EDTA tubes. Samples were transferred to the laboratory within five minutes, centrifuged at $1400 \times g$ at 4°C for 10 min. Samples were aliquoted and stored at -80°C within half an hour after collection.

2.4. Saliva collection

Study 1. Saliva was collected in the morning right before blood collection. Following the recommendations by Mandel and colleagues we used a passive drooling method instead of cotton-based salivettes to collect saliva, because the use of salivettes may result in decreased levels of salivary BDNF (Mandel et al., 2011). Participants were instructed to tilt their head forward and pool saliva in their mouth. When a sufficient amount of saliva was pooled, participants were asked to drool in a cryovial (Salimetrics, Carlsbad, CA, USA). Samples were stored in a cooling bag ($\sim 8^{\circ}\text{C}$) immediately after collection, transferred to the laboratory, centrifuged at $1650 \times g$ at 4°C for 10 min. Samples were aliquoted and stored at -80°C within one hour after collection.

Study 2. Saliva was collected right after blood collection, by passive drooling. Participants were instructed to sit down about 5 min after blood collection and drool into a 20 mL glass vial (PerkinElmer, Waltham, MA, USA) until a 1.5 mL marker was reached. Samples were transferred to the laboratory within five minutes, centrifuged at $1400 \times g$ at 4°C for 10 min. Samples were aliquoted and stored at -80°C within half an hour after collection.

2.5. Procedures ELISA assays

All 33 plasma samples and 33 saliva samples were analyzed in three different commercial ELISA kits: (1) R & D DBD00 (R & D Systems, Minneapolis, MN, USA), (2) LSBio LS-F2402 (LSBio, Seattle WA, USA), and (3) Millipore Chemikine CYT306 (Merck Millipore, Billerica, MA, USA). For sensitivity and detection ranges see Table 2. All kits were validated for human plasma, serum and cell culture supernatants, but not for saliva. Accordingly, the ELISA protocols contained instructions for plasma analyses, but no specific instructions for saliva. Both the

Table 2
Sensitivity and detection ranges of the ELISA kits used in this study, as provided by the manufacturers.

| | Kit 1 | Kit 2 | Kit 3 |
|------------------------------|-----------------|-----------------|-----------------|
| Manufacturer | R & D | LSBio | Merck Millipore |
| Kit type | Sandwich ELISA | Sandwich ELISA | Sandwich ELISA |
| Serial number | DBD00 | LS-F2402 | CYT306 |
| Sensitivity | 20 pg/mL | 31.2 pg/mL | 15 pg/mL |
| Detection range | 62.5–4000 pg/mL | 31.2–2000 pg/mL | 15–1000 pg/mL |
| Intra-assay variation | < 6.2% | < 4.9% | 3.7% |
| Inter-assay variation | < 11.3% | < 7.9% | 8.5% |

R & D kit and the Millipore kit had already been used in previous studies to measure BDNF in saliva (Ikai et al., 2014; Matsuki et al., 2014; Saruta et al., 2012; Tirassa et al., 2012), see also Table 1. The LSBio kit was selected based on the manufacturer's protocol which contained clear instructions and suggested good applicability in our lab.

All plasma analyses were performed according to standard protocol, with the exception that standards and samples were added to the wells in single measures. We did not analyze the samples in duplicate, because we were not interested in the specific individual results but merely in more general patterns of results that could inform us whether BDNF can be quantified in saliva while using commercial ELISA kits according to the manufacturers' protocols. All saliva analyses were performed according to the plasma and serum protocol, with the exceptions that saliva samples were not diluted, and that the saliva standard was based on the diluent that performed best in internal lab tests for constructing saliva standards, i.e., a Tris-NaCl- γ -globuline buffer (0.1 mM Tris/HCl, pH 8.0, 0.1 mM NaCl, 1 g/L bovine γ -globulin (Sigma-Aldrich, St. Louis, MO, USA)). For each kit two sets of standards were used, one for plasma and one for saliva. It was unclear what BDNF concentrations to expect in plasma, therefore different dilutions were used for the plasma samples. For the R & D kit a dilution of 1:20 was used and for the LSBio and the Millipore kit a dilution of 1:2. Since low BDNF concentrations in saliva were expected, saliva samples were not diluted. Weighted 4 parameter logistic (4PL) nonlinear regression models were used for fitting the standard curves. Standard curves are presented together with the results in Fig. 1 (see Results).

2.6. Statistical analyses

Whether the ELISA kits had functioned properly was evaluated by inspecting whether standard curves could be plotted and whether results were within the detection range of the kits. Next, mean BDNF concentrations in plasma and saliva were compared between the three kits, and Spearman correlation coefficients between the different ELISA kits were calculated. Finally, the correlation between BDNF levels in blood and saliva was evaluated.

3. Results

Standard curves could be plotted for BDNF in plasma and in saliva for the R & D and LSBio kits. The Millipore kit could be made operable for neither plasma nor saliva, that is, no standard curve could be plotted. A second attempt with a new Millipore kit yielded the same result. Therefore only the results of the R & D and LSBio kits are provided in this section.

While almost all plasma results lay within the detection range of the R & D and LSBio kit, no saliva sample fell within the range of the calibrated standard curve and the majority did not even exceed the level of the blank (Table 3 and Fig. 1). BDNF in saliva could not be quantified by either kit. The estimated plasma BDNF levels of the two ELISA kits correlated highly ($r_s = .88$, $p < .001$), the mean concentrations differed by almost a factor ten. Because of the lack of valid saliva results it was not possible to calculate the correlation between BDNF in plasma and in saliva.

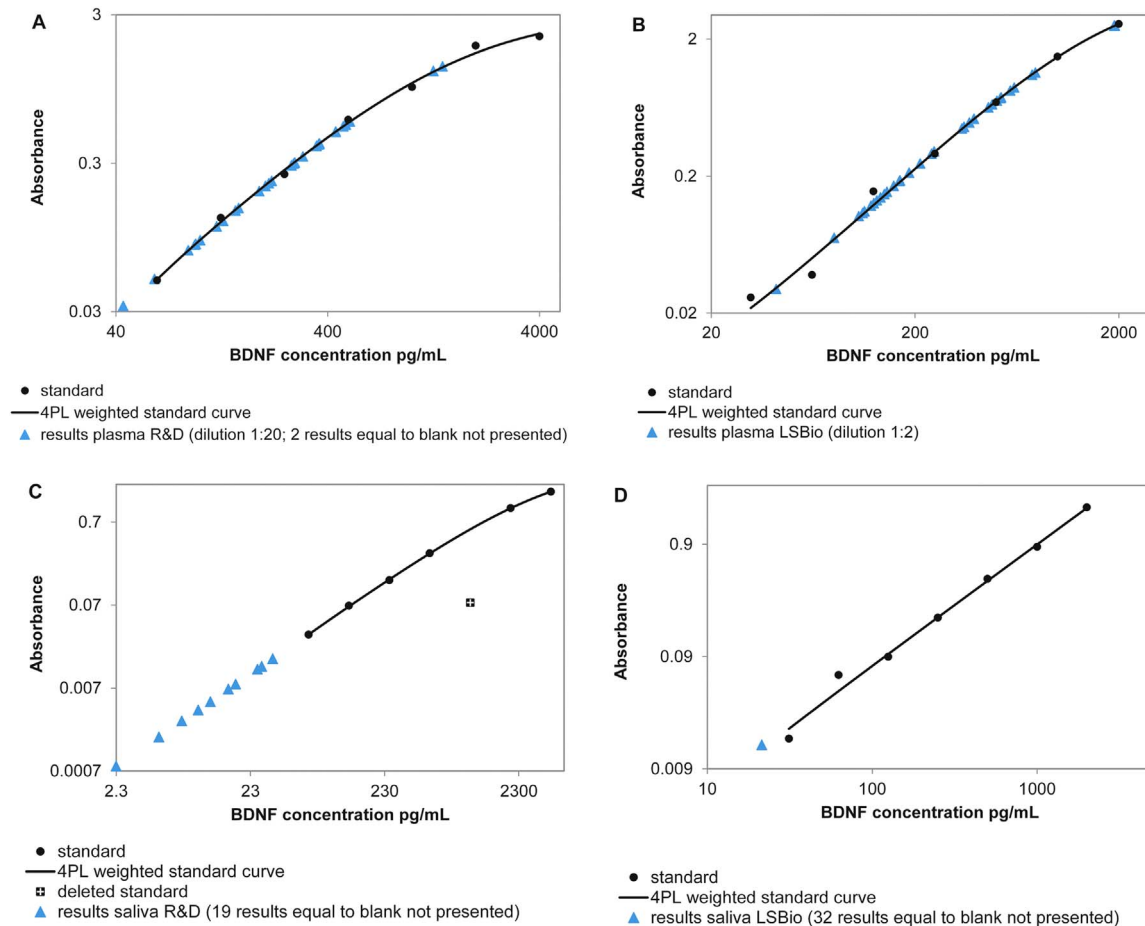


Fig. 1. Standard curves and results of ELISA analyses of BDNF in plasma and saliva. Results of the plasma and saliva analyses are presented for the R & D and LSBio ELISA kits separately. Plasma results are presented in panels A (R & D) and B (LSBio) and saliva results are presented in panels C (R & D) and D (LSBio). Weighted 4 parameter logistic (4PL) nonlinear regression models were used for fitting the standard curves.

Table 3
BDNF in plasma and saliva analyzed in two different ELISA kits.

| | Plasma results within detection range* (%) | Saliva results within detection range* (%) | Saliva results equal to blank | Saliva results above blank but below lowest standard | Mean pg/mL BDNF in plasma (SD) | Mean pg/mL BDNF in saliva (SD) |
|-----------|--|--|-------------------------------|--|--------------------------------|--------------------------------|
| R & D kit | 30 (91%) | 0 (0%) | 19 (58%) | 14 (42%) | 7027 (6065) | – |
| LSBio kit | 33 (100%) | 0 (0%) | 32 (97%) | 1 (3%) | 795 (876) | – |

Each of the 33 plasma and 33 saliva samples was analyzed in both ELISA kits. Mean pg/mL BDNF was calculated only for results within the detection range.

* The detection range is the range of the calibrated standard curve, with as its lower limit the first standard and as its upper limit the highest standard.

4. Discussion

By far most of the saliva samples did not exceed blank level, which means that no BDNF concentrations were measured. The saliva samples that did exceed blank level still did not reach the lowest concentration of the calibrated standard curve and can therefore not be considered reliable. Our data thus suggest that it may be impossible to use commercial ELISA kits according to manufacturers' protocols to reliably quantify BDNF in saliva to date. These results are in agreement with Mandel et al. (2011) but not with de Souza et al., (2014, 2012), Ikai et al. (2014), Tirassa et al. (2012), Saruta et al. (2012) and Matsuki et al. (2014), who reported to have successfully quantified BDNF in saliva using commercial ELISA kits. Differences in interpretation of results below the minimum detection level seem to be at least a partial explanation. Whereas de Souza et al., (2014, 2012), Ikai et al. (2014) and Matsuki (et al. (2014) extrapolated BDNF levels beyond the minimum detection level of the calibrated standard curve, in Mandel et al. (2011) and in our study, BDNF levels beyond the minimum detection level were discarded as unreliable. Saruta et al. (2012) and Matsuki et al. (2014) further reported a minimum detection level of < 4 pg/mL, while the manufacturer's protocol mentions a minimum detection level of 15 pg/mL. Neither Tirassa et al. (2012) nor Saruta et al. (2012) reported on how results below the minimum detection level were handled. It should be noted that the reported mean BDNF levels for healthy young adults varied considerably, even though Saruta et al. (2012) and Matsuki et al. (2014) used the same ELISA kits. Altogether this suggests that commercial BDNF kits may not yet be sufficiently sensitive to measure BDNF reliably in saliva. This is confirmed by the fact that at this moment no ELISA manufacturers have claimed to have validated their kits for measuring BDNF in saliva.

Results for plasma were, as expected, more promising. Almost all results were within the detection ranges of the kits and the high correlation between the two ELISA kits indicates that a similar construct is measured by both. Absolute BDNF levels showed large differences between kits and cannot be compared, because companies use their own BDNF standards and often do not refer to a more objective standard. From the three ELISA kits we assessed, only the R & D protocol contains a statement about the purity of their standard compared to the World Health Organization (WHO) human BDNF standard and how to convert results. To allow for more meaningful interpretations of absolute BDNF levels, it is essential that more manufacturers provide this type of information.

The goal of our study was to assess whether BDNF in saliva can be measured by following standard protocols of commercial kits, without adapting or optimizing the protocol. This is at the same time a strength and a limitation of this study. It is a strength since it mirrors the approach that would often be followed in psychiatric research, yet it leaves unanswered whether we would have succeeded to measure BDNF in saliva if we had experimented with longer incubation times, the amount of sample material, or made other adaptations to manufacturers' protocol. Furthermore, standards and samples were not analyzed in duplo and therefore, although the analysts who conducted the analyses were well-trained, the possibility of pipetting mistakes cannot be excluded. Another limitation is the small number of subjects

participating in our study, i.e., five females and one male. That being said, because of our clear patterns of results for saliva we do not consider it to be likely that our conclusions have been fundamentally influenced by pipetting mistakes and do not expect that including more participants or making minor adaptations to the manufacturers' protocols would change results radically. A final limitation is that the Millipore kit could not be made operable for plasma or saliva. And although this too exemplifies the type of problems encountered when doing this kind of research, it is currently unknown whether this was due to mistakes inside our laboratory or malfunctioning kits.

To conclude, the results of this pilot study tentatively suggest that techniques of commercial BDNF kits are not ready for noninvasive saliva measurements to date, which limits the conditions in which sampling can take place. Because of the benefits of noninvasive and cost effective methods for measuring BDNF, our hopes are that it will be possible to measure BDNF in saliva by using commercial kits in the near future. An alternative approach, which is currently being developed, is to collect blood in less invasive ways (e.g. Hemolink, Tasso Inc, Seattle, WA, USA), which allows the use of commercial ELISA kits that have already been validated for measuring BDNF in plasma and serum. If successful, both approaches are expected to be of great significance to studies in psychiatry.

Author contributions

C. Vrijen^{1,2,3,4,5,6,7}, H.M. Schenk^{2,4,5,6,7}, C.A. Hartman^{4,6,7}, A.J. Oldehinkel^{1,4,6,7}.

¹ conception and design of the study; ² acquisition of data; ³ analysis of data; ⁴ interpretation of data; ⁵ drafting of the article; ⁶ revising the article; ⁷ final approval of the version to be submitted.

Conflicts of interest statement

The authors declare that they have no conflict of interest.

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