[Letter to the Editor]

Influence of clotting duration on brain-derived neurotrophic factor (BDNF) dosage in serum

Brain-derived neurotrophic factor (BDNF) is the most abundant and widely distributed neurotrophin in the central nervous system. BDNF is implicated in several neurodevelopmental functions (1), but it also takes part in nervous system maintenance during adulthood (2).

BDNF is expressed also in peripheral blood, where it is mostly stored in platelets (3); based on this, several studies have evaluated serum BDNF levels in patients affected by different diseases. This research field has been growing considerably in recent years; in particular, alterations of BDNF levels in serum have been described in psychiatric and neurodegenerative diseases. Serum BDNF could therefore represent a valuable peripheral biomarker for such pathologies, with the advantage of being measurable in vivo with noninvasive methods.

However, in a meta-analysis comprising 15 papers on major depression published from 2002 to 2010, we reported that serum BDNF concentrations show a considerable variability between different studies. In healthy subjects, BDNF levels range from 12.2 ± 2.4 [mean $\pm$ standard deviation (SD)] to 64.1 ± 13.1 ng/mL (4). Similar evidence also emerges

from other meta-analyses on bipolar disorder (range: 13.4 ± 0.91 to 42.5 ± 12.5 ng/mL) (5) and anxiety disorders (range: 9.49 ± 3.18 to 41.59 ± 7.82 ng/mL) (6). In addition to nontechnical factors already known to affect serum BDNF levels, such as age, smoking, drinking, fasting, time of sampling, physical activity, and living in an urban environment (7,8), this variability could be attributed to different methodological procedures for serum preparation and storage conditions. Indeed, the duration of serum storage, both at -20°C and -80°C , was demonstrated to be inversely correlated with measured BDNF levels, due to the progressive activity of proteolytic enzymes (9,10). Furthermore, the duration and temperature of clotting and the duration and speed of centrifugation, which are highly variable between different studies, are likely to affect the measurement of BDNF levels. In particular, because BDNF is stored in platelets, clotting duration could constitute a critical issue for its dosage in serum. In the studies published so far, the clotting duration varies from 30 min to 3 hr, when specified, but it is often not clearly stated (expressions such as “blood was allowed to clot” are used). The only study that evaluated this issue was conducted in

rats by measuring serum BDNF after increasing clotting durations. Stable BDNF levels were described from 1 to 24 hr of clotting, but earlier time points were not evaluated (11).

Due to the preceding concerns, in the present study we investigated the influence of clotting duration on BDNF levels in human serum, both at early and late time points, to determine the time course of BDNF release from platelets and to help to establish a standardized procedure for its dosage.

Ten healthy volunteers (8 females and 2 males, mean age 32.3 ± 7.3 years) were enrolled in the study, and 5 venous blood samples for each subject were drawn in the morning in anticoagulant-free tubes. Each tube was kept at room temperature for 10 min, 30 min, 1 hr, 2 hr, or 5 hr. For 5 subjects, a sixth sample was collected to test clotting conditions of 1 hr at room temperature followed by 1 hr at 4°C . Serum separation was subsequently obtained by centrifugation at $1620 \times g$ for 15 min at room temperature. Serum samples were immediately stored at -80°C until the time of assay. BDNF levels were measured within 1 month with enzyme-linked immunosorbent assay (ELISA) (BDNF DuoSet kit, R&D Systems, Minneapolis, MN), in duplicate. BDNF content was

Table 1. Serum BDNF measurement values at different clotting durations and means $\pm$ standard deviations (ng/mL).

Subject Id/Clotting duration	10 min	30 min	1 hr	2 hr	5 hr	Mean 1 hr-2 hr-5 hr $\pm$ SD
1	9.47	30.04	32.94	31.14	31.49	31.86 ± 0.95
2	10.30	35.43	33.36	33.34	33.50	33.4 ± 0.09
3	14.11	28.27	29.46	29.77	30.33	29.85 ± 0.44
4	17.05	29.48	30.93	34.26	34.29	33.16 ± 1.93
5	29.04	42.20	47.94	44.29	42.87	45.04 ± 2.62
6	16.38	26.33	34.56	32.23	31.25	32.68 ± 1.7
7	14.98	33.13	32.73	31.54	33.10	32.46 ± 0.82
8	5.31	24.64	34.80	34.61	31.70	33.7 ± 1.73
9	6.65	28.26	33.65	28.05	28.60	30.1 ± 3.08
10	9.84	40.91	45.68	47.22	42.11	45 ± 2.62
Mean $\pm$ SD	13.31 ± 6.82	31.87 ± 5.97	35.61 ± 6.14	34.65 ± 6.22	33.92 ± 4.8	34.73 ± 5.58

expressed as nanograms of protein per milliliter (ng/mL) of serum, taking into account the dilution factor used (1:100), with a detection limit of 20 pg/mL.

The mean BDNF concentration determined in serum after 10 min of clotting at room temperature was 13.31 ± 6.82 ng/mL, increasing to 31.87 ± 5.97 ng/mL after 30 min and to 35.61 ± 6.14 ng/mL after 1 hr. Thereafter, the measurements appeared stable: 34.65 ± 6.22 and 33.92 ± 4.8 ng/mL after 2 hr and 5 hr, respectively. For the further clotting condition of 1 hr at room temperature followed by 1 hr at 4°C, the results were strictly similar to those obtained after 1 hr at room temperature (data not shown). To obtain a statistical assessment of these variations, a repeated-measures analysis of variance (rANOVA) was performed through the Statistical Package for the Social Sciences (SPSS) Version 17 (IBM Corp., Armonk, NY), comparing concentrations at 10 min, 30 min, 2 hr, and 5 hr to concentrations at 1 hr. Significant differences were observed at 10 min ($P = 2.97 \times 10^{-6}$) and 30 min ($P = 0.01$), whereas no modifications were evident at 2 hr ($P = 0.27$) and 5 hr ($P = 0.09$). The mean value ($\pm$ sd) of the quantifications at 1 hr, 2 hr, and 5 hr was 34.73 ± 5.58 ng/mL; compared to this plateau value, the mean levels at 10 min and 30 min were 38% and 91.8%, respectively. Single-subject measurement values and means ($\pm$ sd) are reported in Table 1. In Figure 1A, the time course of BDNF level for every subject is shown, whereas the mean BDNF concentration ($\pm$ sd) for every clotting duration is represented in Figure 1B.

Peripheral BDNF represents a potentially highly valuable biomarker, which could help in the diagnosis and prognosis of psychiatric and neurodegenerative diseases, as well as in the prediction of treatment response and the evaluation of therapeutic effectiveness. To date, more than 400 articles have been published investigating serum BDNF levels in different pathological conditions. A major issue with these studies concerns the methodological aspects, because different meta-analyses reported a

considerable variability in serum BDNF concentrations in healthy subjects between different studies (4–6), possibly due to different technical procedures for serum preparation and storage conditions. Because BDNF is stored in platelets, the main variable likely to affect BDNF dosage is the duration of the clotting process. We have investigated the influence of increasing clotting durations at room temperature, observing a progressive enhancement of serum BDNF content: the concentrations reached 38% of the plateau value after 10 min, 91.8% after 30 min, and 100% after 1 hr, probably reflecting the kinetics of BDNF release from platelets; additional resting for 1 hr at 4°C had no effect. Therefore, we recommend 1 hr as the minimum clotting duration for a correct serum BDNF dosage.

Author contributions

L.B.-C., E.M. and M.G. designed the experiment. E.M. and R.Z. arranged

and performed blood collection, serum preparation, and BDNF measurement. E.M. and L.B.-C. analyzed the results. E.M. and L.B.-C. wrote the paper. M.G. and R.Z. revised the paper.

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Competing interests

The authors declare no competing interests.

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Figure 1. Influence of clotting duration on BDNF concentration. (A) Time course of BDNF concentration for every subject. (B) Mean BDNF concentration $\pm$ standard deviation for every clotting duration.

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