DETECTION OF MACROPROLACTINEMIA AND MOLECULAR CHARACTERIZATION OF PROLACTIN ISOFORMS IN BLOOD SAMPLES OF HYPERPROLACTINEMIC WOMEN

OTKRIVANJE MAKROPROLAKTEMIJE I MOLEKULARNA KARAKTERIZACIJA IZOFORMI PROLAKTINA U UZORCIMA KRVI HIPERPROLAKTINEMIČNIH ŽENA

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Summary: Prolactin (PRL) circulates in the blood in the form of monomeric prolactin, dimeric prolactin and macroprolactin. Macroprolactin is a common cause of hyperprolactinemia. The objective of this study was to assess the prevalence of macroprolactinemia in hyperprolactinemic women and to undertake the biochemical characterization of macroprolactin. A retrospective cross-sectional study was conducted on one hundred hyperprolactinemic patients. All the sera were subjected to polyethylene glycol (PEG) precipitation and were divided into true hyperprolactinemics (PRL recovery >60%), probable macroprolactinemics (PRL recovery between 40 and 60%) and macroprolactinemics (PRL recovery < 40%). The prevalence of macroprolactinemia was found to be 34%. Sera from each group were further analyzed for isoforms of prolactin by gel filtration chromatography (GFC). The clinical spectrum of presenting complaints in the hyperprolactinemic cohort included oligomenorrhea, galactorrhea and infertility, but the presentation did not differ between macroprolactinemic and truly hyperprolactinemic patients. GFC showed three major PRL isoforms, viz., 23.5 kDa (monomeric), 47 kDa (dimeric) and 150–174.6 kDa (PRL–IgG complexes) along with the medium and heavy weight aggregates of prolactin. The results of the study showed that macroprolactinemia is one of the causes of hyperprolactinemia with high prevalence. It is recommended that all hyperprolactinemic patients be screened for macroprolactinemia.

Keywords: macroprolactin, pseudohyperprolactinemia, big-big prolactin, IgG-prolactin, hyperprolactinemia, macroprolactinemia

Kratak sadržaj: Prolaktin (PRL) cirkuliše kroz krvotok u obliku monomernog prolaktina, dimernog prolaktina i makroprolaktina. Makroprolaktin često izaziva hiperprolaktinemiju. Cilj studije bio je da se utvrdi prevalenca makroprolaktinemije kod žena sa hiperprolaktinemijom i da se izvrši biohemijska karakterizacija makroprolaktina. Sprovedena je retrospektivna studija preseka koja je obuhvatila stotinu pacijenata sa hiperprolaktinemijom. Svi uzorci seruma podvrgnuti su precipitaciji polietilen-glikolom (PEG) i podeljeni na prave hiperprolaktinemične (PRL >60%), verovatno makroprolaktinemične (PRL između 40 i 60%) i makroprolaktinemične (PRL < 40%). Utvrđena prevalenca makroprolaktinemije bila je 34%. U uzorcima seruma svih grupa ispitivano je prisustvo izoformi prolaktina putem hromatografske filtracije gelom (GFC). Prijavljene kliničke smetnje u skupini pacijenata sa hiperprolaktinemijom obuhvatile su oligomenoreju, galaktoreju i neplodnost, ali se prezentacije nisu razlikovale između pacijenata sa makroprolaktinemijom i pravih hiperprolaktinemičnih pacijenata. GFC je pokazala tri glavne izoforme PRL, naime 23,5 kDa (monomer), 47 kDa (dimer) i 150–174,6 kDa (kompleksi PRL–IgG) zajedno sa srednje i veoma teškim agregatima prolaktina. Rezultati studije pokazali su da je makroprolaktinemija jedan od uzroka hiperprolaktinemije koji ima veliku prevalencu. Preporučuje se obavljanje pregleda za makroprolaktinemiju kod svih pacijenata sa hiperprolaktinemijom.

Ključne reči: makroprolaktin, pseudohiperprolaktinemija, big-big prolaktin, IgG–prolaktin, hiperprolaktinemija, makroprolaktinemija

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Introduction

Hyperprolactinemia is one of the most common endocrine disorders of the hypothalamic–pituitary axis. In women, the disorder is characterized by galactorrhea, infertility and menstrual disturbances. Hyperprolactinemic men present with hypogonadism, oligospermia, gynecomastia, lack of libido and impotence (1). However, hyperprolactinemia could also be caused by a circulating isoform of prolactin which is higher in molecular weight and has a much longer biological half-life in plasma. This large prolactin (bbPRL) predominates in some patients with hyperprolactinemia, and the condition is called macroprolactinemia or pseudohyperprolactinemia (2). In this condition, the patient's serum harbours macroprolactin with a molecular mass greater than 150 kDa in addition to monomeric 23 kDa PRL (3).

The circulating isoforms of plasma prolactin can be categorized into monomeric prolactin (mPRL, MW, 23 kDa), big prolactin (bPRL, MW 40–65 kDa), and big-big prolactin or macroprolactin (bbPRL) with a molecular weight between 150 and 170 kDa (4). Monomeric prolactin, 23 kDa, is the biologically active form of prolactin and contributes more than 80% to the total serum prolactin in a majority of normal and hyperprolactinemic individuals. The mPRL is responsible for the physiological activity and pathological symptoms due to prolactin hormone. Macroprolactin or IgG-bound prolactin isoforms have no clinical importance because they exhibit little biological activity (5). Although biologically inactive or minimally active, macroprolactin is immunoreactive in most of the immunoassays used for the estimation of serum prolactin levels and thus can cause a misdiagnosis of hyperprolactinemia (6). The frequency and clinical consequences of macroprolactinemia have not been clearly established, mainly because of difficulty in identifying the patients, clinically as well as biochemically (7, 8).

Gel filtration chromatography (GFC) is the gold standard method for quantifying mPRL and bbPRL in sera but it is expensive and labor-intensive. Fractionation of bbPRL by precipitating with polyethylene glycol (PEG) is a simple, rapid and inexpensive method as compared to GFC and is suitable for routine application (7).

If macroprolactinemia is proved to be a separate, milder entity from true hyperprolactinemia, its identification in the patient's plasma can prevent unnecessary and expensive pituitary imaging and inappropriate medication and surgical intervention. The present study looks into the prevalence of macroprolactinemia in the hyperprolactinemic individuals and attempts to study the circulating isoforms of prolactin in plasma.

Materials and Methods

Study subjects

Sera from 611 patients referred to the Ethiopian Health and Nutrition Research Institute (EHNRI) were assayed for PRL with the electrochemiluminescence immunoassay (ECLIA) system. Based on the cut-off values provided by the manufacturer of ECLIA kits, 511 patients (83.6%) were found to be normo-prolactinemic, while 100 (16.4%) were hyper-prolactinemic. Sera from the hyperprolactinemic individuals were used for the identification and characterization of the circulating isoforms of prolactin.

Methods

PEG precipitation of macroprolactin. The serum sample (200 μL) was mixed with an equal volume of 25% PEG solution. The mixture was thoroughly vortexed and allowed to stand for 20 minutes. After centrifugation at 2500 × g for 15 minutes, the supernatant was aspirated and analyzed for PRL concentration in the same way as the original samples. The precipitate was resuspended in 1 mL of 0.02 mol/L phosphate buffer and stored at 4 °C for further analysis. Ratio of the PRL in the supernatant to total PRL in serum was calculated and presented as the percentage recovery of PRL. As recommended by Fahie Wilson and Ahlquist (2003), the samples were classified into truly hyperprolactinemic (recovery >60%), probably macroprolactinemic (recovery 40–60%) and macroprolactinemic (recovery <40%).

Gel Filtration Chromatography. A column packed with Seralose-6B® (Sisco Research Laboratories, Mumbai, India) was used for the fractionation of serum proteins. Selected serum samples and resuspended precipitates (170 μL) diluted with 130 μL of phosphate buffer (pH 7.4) were loaded on the column and eluted with phosphate buffer. Fraction collection was started after the void volume (V_o) of 19 mL. A constant flow rate of 16 drops/minute was maintained and 1.5 mL fractions were collected up to a total volume of 57 mL. Every two consecutive fractions were pooled and their protein content estimated by measuring their optical density (OD) at 280 nm. A graph of OD against elution volume (V_e) was plotted to identify the protein fractions.

Prolactin concentration was estimated in all the fractions and another graph of prolactin concentration (ng/mL) against V_e was plotted. The relative area under peaks of prolactin isoforms was calculated. A standard curve of Log MW vs. V_e/V_o was plotted and molecular weight of the prolactin isoforms estimated against the standard molecular weight markers.

Prolactin estimation. Prolactin concentration in the patients' sera or in the GFC fractions was measured by an electrochemiluminescence immunoassay on the Roche Elecsys 2010 system.

Statistical analysis. The data generated from the study were analyzed using the Epi-Info 2000 statistical package for epidemiological research. The p-values below 0.05 were considered as significant. The graphs and figures were generated by using Microsoft Excel (MS Office 2007).

Ethical consideration

Before the beginning of the research project, clearance was obtained from the Ethical Committee of the Faculty of Medicine, AAU.

Results

Prevalence of macroprolactinemia

The study subjects showed a wide variation in the prolactin values, ranging from 16.5 to 4.672 ng/mL. They were screened for macroprolactinemia with the help of PEG precipitation. Among the hyper-prolactinemic patients, 34 patients showed a recovery of less than 40%, i.e. the prevalence of macroprolac-tinemia in our study group (hyperprolactinemia) was 34% of patients (*Figure 1*). A recovery between 40% to 60% was observed in 16% of the cases, and these patients were, therefore, labeled as 'probably macro-prolactinemic'; the rest of the patients (50%) were identified as truly hyperprolactinemic subjects.

The macroprolactinemic group was composed of patients of age ranging from 19 to 65 years with an average (mean ± SEM) of 32.9 ± 1.4 yrs. Preva-lence of macroprolactinemia was a little lower in the younger (less than 25 year) age group (*Table I*); whereas above 35 years of age, the prevalence was almost constant (around 35%). Females above 40 years of age showed significantly (p<0.05) higher prevalence of macroprolactinemia.

Similarly, we analyzed the variation in macro-prolactinemia among the patients with increasing serum concentrations of prolactin to define a cut-off prolactin level above which sera should be screened for macroprolactin. It was observed that macro-lactinemia was more prevalent in patients having high-er serum prolactin levels than in those with prolactin values near the normal ranges. The data (*Figure 2*) demonstrated that the serum prolactin level of 120 ng/mL could be taken as cut-off, i.e. significantly high-er prevalence (56.3%) was seen in patients with higher prolactin levels than in those with prolactin levels less than 120 ng/mL (27.6%). The average mean ± SEM prolactin levels in the macroprolactinemic and truly hyperprolactinemic groups were 111.9 ± 16.6 ng/mL and 25.7 ± 3.9 ng/mL, respectively.

Table I Prevalence of macroprolactinemia among different age groups.

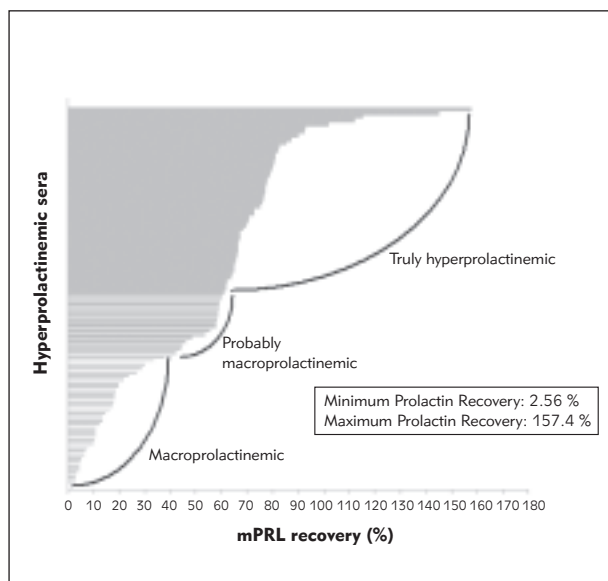| Age (yrs.)	Macroprolactinemia (%)
<25	15.4
25–29	36.4
30–34	35.7
35–39	26.3
40–44	55.6*
>44	0.0

* p< 0.05

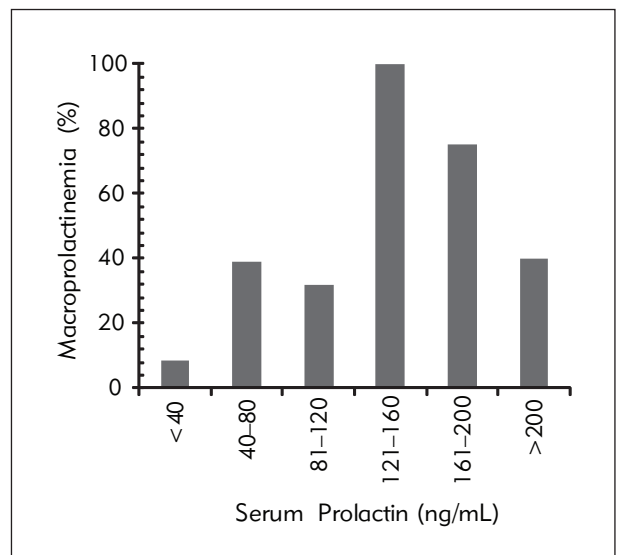**Figure 1** Identification of macroprolactinemia.

Figure 2 Incidence of macroprolactinemia at different concentrations of serum prolactin.

Clinical presentation

The clinical symptoms of the patients with macroprolactinemia did not differ from the truly hyperprolactinemic patients. There are no clinical features that could reliably differentiate macroprolactinemia from true hyperprolactinemia. Oligomenorrhea was the most frequent complaint of the patients in both groups (81.8% in macroprolactinemic and 68.4% in truly hyperprolactinemic patients). Galactorrhea was the chief complaint in 19.7% of macroprolactinemic and 17.3% of truly hyperprolactinemic patients. Out of the seven patients who were referred for infertility, only 2 had macroprolactinemia.

Biochemical characterization of the sera

Gel filtration chromatography revealed the existence of multiple isoforms of prolactin in circulation in almost all the sera tested. In the truly hyperprolactinemic group, the elution pattern showed that all the sera contained a prolactin isoform with a molecular weight of 23.5 kDa (*Figure 3a*). Another elution peak was observed at approximately 47 kDa (*Figure 3b*) in some of the sera and the proportion of the two fractions varied in different sera.

The GFC pattern of the macroprolactinemic sera showed a peak around 150 kDa and a shoulder around 23.5 kDa (*Figure 4a*). At least seven (20.6%) of the macroprolactinemic sera showed the presence of a very high molecular weight (~240 kDa) isoform of prolactin (*Figure 4b*).

One serum from the third group, i.e. 'probably macroprolactinemic' patients, was also analyzed by gel filtration – it showed an intermediate pattern (*Figure 4c*) with the GFC pattern showing prolactin peaks at 23.5, 69.3 and 174.6 kDa.

The GFC pattern of PEG precipitates resuspended in buffer showed a major prolactin peak that eluted at around 150 kDa.
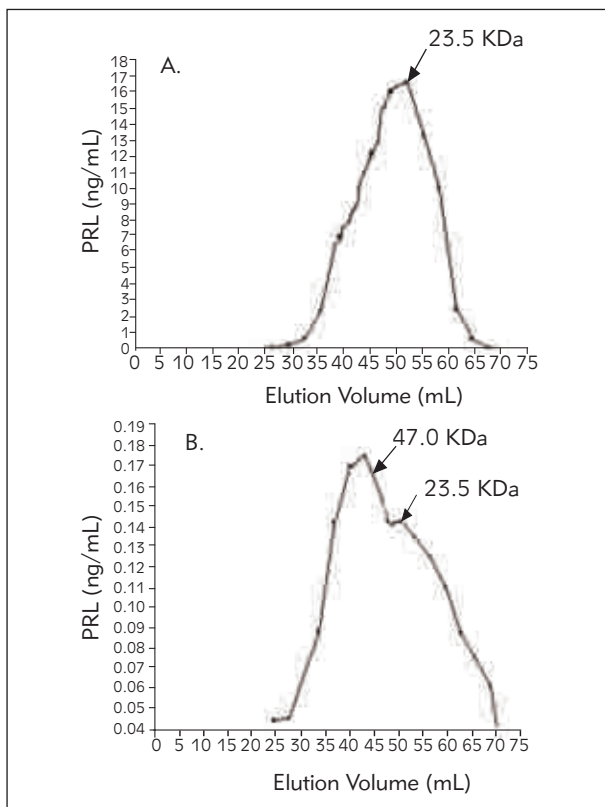

Figure 3 Isoforms of circulating prolactin – A. GFC elution pattern of a truly hyperprolactinemic serum; B. GFC elution pattern of a truly hyperprolactinemic serum showing a dimeric form of prolactin.
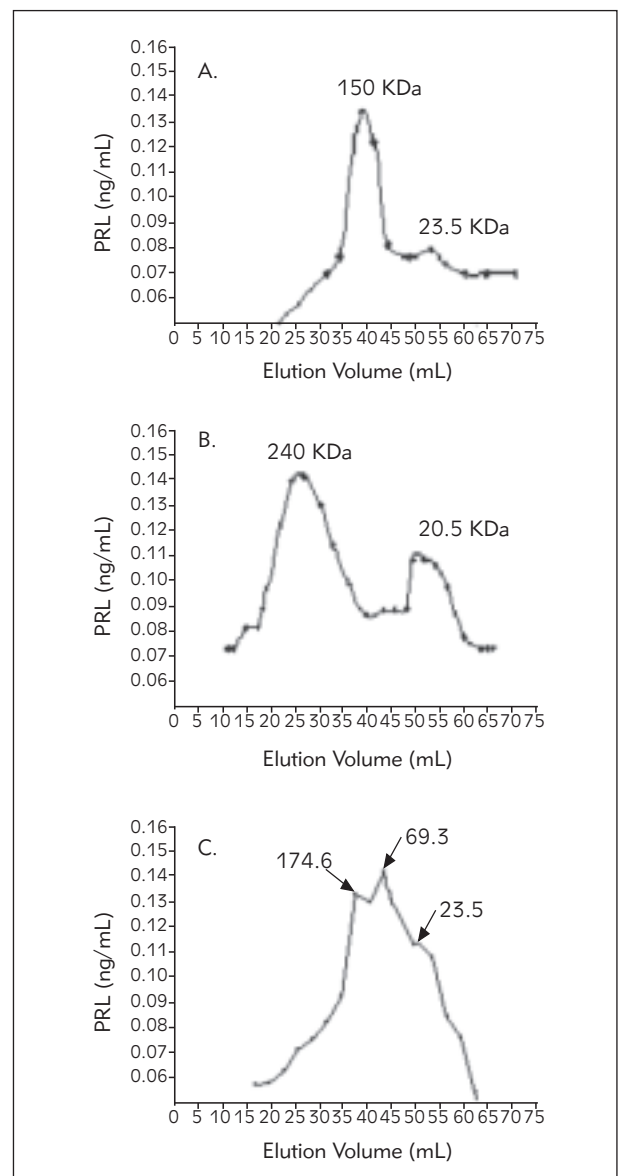

Figure 4 A polymorphism of circulating prolactin – A. & B. GFC elution pattern of macroprolactinemic sera; C. GFC elution pattern of a 'probably macroprolactinemic' serum.

Discussion

Detection and prevalence of macroprolactin

The polyethylene glycol (PEG) precipitation has become the method of choice to detect pseudo-hyperprolactinemia caused by big prolactin and/or macroprolactin. We used PEG precipitation to screen for macroprolactinemia among the referred hyper-prolactinemic patients in our laboratory. Macroprolactin reacts very strongly in the Roche Elecsys 2010 electrochemiluminescence system used in our study and is a common cause of hyperprolactinemia (90). We used a 40% cut-off of prolactin recovery after PEG precipitation for the identification of macroprolacti-nemia. Prevalence of macroprolactinemia in the hyper-prolactinemic individuals was 34%. Majority of the hyperprolactinemic patients referred to our laboratory were females and among these the prevalence of macroprolactinemia was 32.6%. On the other hand, only eight male subjects were referred to our laboratory and amongst these four (50%) showed macro-prolactinemia.

The prevalence as well as clinical manifestation of macroprolactinemia has been studied by a number of research groups. In the general adult population, hyperprolactinemia is prevalent in about 0.4% of individuals, but among hyperprolactinemic patients, macroprolactinemia could be seen in about 22% of the cases in the USA (10). Hattori et al. (11) reported macroprolactinemia in 3.68% of the healthy hospital workers and 30.6% of hyperprolactinemic patients. Reports from different regions across the globe have put the prevalence of macroprolactinemia between 10% and 46% (12). The prevalence of 34% for macro-prolactinemia among hyperprolactinemic patients in the present study is consistent with these reports. In our own study conducted in India (unpublished work), we observed the prevalence of only 11% in hyperprolac-tinemic females. Ours is the first report on macropro-lactinemia in the African population and the higher prevalence could have an ethnic basis.

One of the probable causes of the higher preva-lence of macroprolactinemia in our subjects could be the co-precipitation of mPRL with bbPRL, which might have led to lower per cent recovery, causing us to over-report macroprolactinemia. Suliman et al. (1) suggested that normal sera treated with PEG can be used as »blanks« whose bbPRL values could be subtracted from bbPRL values of hyperprolactinemic sera treated the same way. We tried a similar method and used sera from healthy normal (non-hyperpro-lactinemic) volunteers as blanks, but bbPRL preci-pitated by PEG from these sera was found to be too low (<2.0%) to affect the results and hence was not used in the calculation of recovery. Another reason for not using such blanks was, in our opinion, these sera would not represent true blanks since the immu-noglobin concentration varies in sera from different individuals.

The major form of macroprolactin is IgG-bound prolactin. We tried to relate the incidence of macro-prolactinemia with age of the patient, with the idea that the prevalence of autoimmunity and hence anti-prolactin antibodies would increase with age. Our re-sults did not corroborate this assumption and macro-prolactinemia was found to be almost similar at all the ages in the fertility age groups. Leslie et al. (2) had also observed macroprolactinemia in a wide range of females i.e. from 18 to 55 years. It appears that auto-immunity plays no role in the development of macro-prolactinemia (11).

Isoforms of prolactin and confirmation of macroprolactinemia

The prolactin elution patterns from other studies typically show two (13) or three (8) discrete peaks for mPRL, bPRL and bbPRL.

Smith and Norman (14) were the first to cal-culate the molecular weights of the isoforms of PRL circulating in the blood. They documented the mole-cular weights of different isoforms of prolactin ranging from 23 kDa to 170 kDa. A 50–60 kDa pro-lactin isoform is found between these low and high molecular weight isoforms. According to Gibney et al. (3), 150–200 kDa prolactin isoforms are consistent with the concept of a PRL–immunoglobulin complex.

In the present study, we were able to differenti-ate the macroprolactinemic from the truly hyper-prolactinemic sera by sepharose gel filtration. The macroprolactin (IgG bound prolactin) typically eluted at a molecular weight of 150 kDa and was the pre-dominant isoform of prolactin in these sera. The point of elution for bbPRL was determined by comparing the prolactin peaks in a macroprolactinemia serum and the PEG precipitate (suspended in phosphate buffer); a 150 kDa peak for PRL was seen in both these solutions.

We calculated areas under the curves to estimate the proportion of macroprolactin in the total prolactin eluted from the column. A sample is classified as being macroprolactinemic when the proportion of bbPRL present, as determined by analysis of the area under the PRL curve, is > 50–60% of the total area of PRL elution. In most of the macroprolactinemic sera in our study, the bbPRL peak contributed about 50–72% to the eluted prolactin. Our results are in agreement with those of Fahie-Wilson and Ahlquist (15).

However, most of the sera even from macro-prolactinemic patients showed a peak at 23.5 kDa, i.e., the monomeric prolactin exists in the presence of bbPRL and is in equilibrium with the IgG-bound pro-lactin. The mPRL circulates along with bbPRL in these patients and could be the product of dissociation of bbPRL *in vivo*, i.e., bbPRL acts like a reservoir of biologically active prolactin and is in equilibrium with

the unbound prolactin. Hattori et al. (16) have suggested that mPRL could arise from the dissociation of prolactin from IgG during GFC. The equilibrium in such a situation would be determined by the affinity of the antibodies for prolactin molecule.

The GFC of sera from patients with equivocal post PEG recovery (40–60%) displayed an equally ambiguous pattern of prolactin elution. A prolactin peak was noticed between mPRL and bbPRL in most of these patterns, and eluted at about 65 kDa. The molecular weight indicates dimerization of prolactin to give rise to this third isoform, i.e. bPRL. A number of these 'probably macroprolactinemic' sera displayed all the three peaks. Since neither mPRL nor bPRL is precipitated with PEG, the overall post-PEG prolactin recovery is more than 40% even in the presence of macroprolactin. The presence of this dimeric bPRL has been reported by a number of studies. Smith and Fahie-Wilson (5) have also emphasized the existence of multiple forms of prolactin in circulation including 23 kDa mPRL, 60 kDa bPRL and 150 kDa bbPRL. Big prolactin (60 kDa) and macroprolactin (150 kDa), which are present in serum in varying quantities, can cause apparent hyperprolactinemia. Since it seldom exists in the absence of bbPRL, the biological activity of bPRL has not been documented very well. Theoretically, bPRL could have intermediate biological activity and cause mixed clinical symptoms.

Hattori et al. (11) have attempted to characterize the bbPRL. They investigated the presence of specific antibodies against prolactin to form IgG-bound macroprolactin. The levels of IgG-bound prolactin positively correlated with those of macroprolactin, suggesting that IgG-bound prolactin forms a major share of macroprolactin. Approximately three quarters of the subjects with macroprolactinemia displayed the presence of anti-prolactin autoantibodies. However, they also suggested that macroprolactin is not only formed of specific PRL-Ab interactions, but glycosylation, aggregation and covalent/noncovalent binding were also involved in the formation of macroprolactin. The prolactin aggregates could be responsible for the very high molecular weight (240 kDa) prolactin seen in some macroprolactinemic individuals.

Clinical significance

The clinical significance of macroprolactinemia has been an issue of debate for many years. Some reports have associated it with galactorrhea, menstrual irregularities and infertility, whereas others have suggested that it causes no symptoms. Macroprolactin has been reported to be biologically inactive and hence macroprolactinemia should not present with any clinical symptoms. However, Alfonso et al. (10) have reported menstrual disturbance in 56% of macroprolactinemic females. We compared the clinical features of the two groups of patients and found that the incidence of oligomenorrhea (81.8%) was non-significantly higher in the macroprolactinemic females compared to 68.4% seen in the true hyperprolactinemia group. Galactorrhea was almost as common in both the groups. The incidence of galactorrhea observed in our study compares well with that of Alfonso et al. (10) but is lesser than that reported by Vallette-Kasic et al. (17). The higher incidence of oligomenorrhea in our study group may be due to the types of patients referred to our laboratory – most of the cases were chronic cases since unavailability of the reliable laboratories in Ethiopia is responsible for delayed diagnosis and slower treatment. The prolonged delay and hence chronic exposure to high prolactin levels could trigger the immune response and generate macroprolactin. In our study, the macroprolactinemia cases could not be differentiated from true hyperprolactinemia on the basis of clinical features. The presence of clinical features in equal or even higher proportion could also be attributed to the possible dissociation of prolactin from low affinity but high capacity IgG antibodies in macroprolactin that could lead to increased bioavailability of monomeric prolactin (18, 19). Owing to its high molecular mass, macroprolactin, as such, appears to be confined to the intravascular compartment and much evidence indicates that it has minimal bioactivity *in vivo* and is not of pathological significance (20). However, the variance in the clinical features of macroprolactinemia could be due to the differences in the affinity of the antibodies for prolactin. The low affinity could result in increased concentrations of biologically active prolactin under different physiological conditions, thus manifesting as clinical symptoms of hyperprolactinemia.

The management of macroprolactinemia has also been a topic of controversy. Some studies have reported that dopaminergic treatment results in an improvement in the hyperprolactinemic symptoms along with decrease in macroprolactin (21). However, in the only progressive study available on macroprolactinemia, Wallace et al. (22) have reported that macroprolactinemia is a benign condition and apart from the oligomenorrhea and galactorrhea in some patients, no further deterioration in clinical condition was observed during a 10 year follow up of macroprolactinemic patients. Therefore, once the patient is identified with macroprolactinemia, extended endocrine review or a follow up is not required.

Conclusions and Recommendations

Macroprolactinemia is as relevant in Africa as it is in anywhere else in the world. Failure to recognize macroprolactinemia as a cause of hyperprolactinemia leads to unnecessary investigations, incorrect diagnosis and inappropriate treatment. Despite efforts to improve assay specificity for mPRL alone, all prolactin immunoassays, currently in use, detect both big prolactin and macroprolactin to varying degrees.

Monomeric prolactin and big-big prolactin are distinct entities and the variation in their proportions in different patients could lead to different clinical presentations. PEG pre-treatment of sera is a useful method for the screening of macroprolactinemia and gel filtration chromatography proved to be a reliable method for confirmation of macroprolactinemia.

It is thus recommended that all sera with increased total prolactin concentrations (>120 ng/mL) be sub-fractionated by PEG precipitation to measure the bioactive monomeric prolactin concentration. The method is cost effective and easily available in all the endocrinology laboratories.

Conflict of Interests

None. No pharmaceutical/diagnostic company was involved in the funding of this project.

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