

# Hyperprolinemia in Type 2 Glutaric Aciduria and MADD-Like Profiles

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**Abstract** Classical neonatal-onset glutaric aciduria type 2 (MAD deficiency) is a severe disorder of mitochondrial fatty acid oxidation associated with poor survival. Secondary dysfunction of acyl-CoA dehydrogenases may result from deficiency for riboflavin transporters, leading to severe disorders that, nevertheless, are treatable by riboflavin supplementation. In the last 10 years, we identified nine newborns with biochemical features consistent with MAD deficiency, only four of whom survived past the neonatal period. A likely iatrogenic cause of riboflavin deficiency was found in two premature newborns having parenteral nutrition, one of whom recovered upon multivitamin supplementation, whereas the other died before diagnosis.

Four other patients had demonstrated mutations involving ETF or ETF-DH flavoproteins, whereas the remaining three patients presumably had secondary deficiencies of unknown mechanism. Interestingly, six newborns among the seven tested for plasma amino acids had pronounced hyperprolinemia. In one case, because the initial diagnostic workup did not include organic acids and acylcarnitine profiling, clinical presentation and hyperprolinemia suggested the diagnosis. Analysis of our full cohort of >50,000 samples from >30,000 patients suggests that the proline/alanine ratio may be a good marker of MAD deficiency and could contribute to a more effective management of the treatable forms.

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## Introduction

Multiple acyl-CoA dehydrogenase deficiency (MADD), also known as glutaric aciduria type 2 (GA2, OMIM 231680), is a rare autosomal recessive metabolic disease, caused by defects in the mitochondrial electron transfer flavoprotein (ETF) or, in its electron acceptor counterpart, the electron transfer flavoprotein dehydrogenase (ETF-DH or ETF-QO) (Frerman and Goodman 1985). Neonatal-onset forms are characterized by nonketotic hypoglycemia, metabolic acidosis, hepatomegaly, and hypotonia, usually with fatal outcome during the neonatal period or the first months of life (Frerman and Goodman 2001). A few of these patients have congenital anomalies, such as renal cystic dysplasia, facial dysmorphism, rocker bottom feet, and abnormalities of external genitalia (Frerman and Goodman 2001), while the others often develop severe cardiomyopathy during the neonatal period. Cases of

secondary MADD related to riboflavin deficiency have been described in neonates of mothers carrying heterozygous mutations in a riboflavin transporter (deficiency for GPR172B/SLC52A1) and showed a good response to transient riboflavin supplementation (Chiong et al. 2007; Harpey et al. 1983; Ho et al. 2011). A different entity with later presentation, Brown–Vialeto–Van Laere syndrome, is caused by mutations in two related riboflavin transporters SLC52A2 and SLC52A3 (Bosch et al. 2011; Green et al. 2010; Johnson et al. 2012).

Important biochemical anomalies guide the diagnosis of primary or secondary MADD. Plasma acylcarnitine profiles show an increase of all chain-length acylcarnitines, while urine organic acid profiles display high concentrations of glutaric, 2-hydroxyglutaric, ethylmalonic and other dicarboxylic acids, and several glycine derivatives (Frerman and Goodman 2001). Early studies reported that a marked increase of plasma proline is common in neonatal-onset patients (Frerman and Goodman 2001; Goodman et al. 1983; Przyrembel et al. 1976; Sweetman et al. 1980), but plasma amino acid analysis does not usually play a part in the diagnostic workup.

In this report, we have identified nine newborns with biochemical features consistent with primary or secondary MAD deficiency in our local cohort and investigated the potential clinical interest of amino acid analyses in this context by comparison to a large cohort of patients.

## Subjects and Methods

Between 2003 and 2014, we have identified four neonates with primary MAD deficiency and five others with suspected secondary MAD deficiency. Of these, 1/4 and 3/5 patients, respectively, survived past the neonatal period. Table 1 summarizes the relevant clinical and biochemical features and further details are presented in this section.

We observed primary neonatal MADD in four newborns (patients 1–4). Patients 1 and 2 showed typical neonatal MADD onset with hypoglycemia, metabolic acidosis with hyperlactatemia, mild hyperammonemia, hepatomegaly and hypotonia, and classical biochemical profiles associating very high glutaric levels (7,080 and 5,280 mmol/mol creatinine, respectively) with increased ethylmalonic acid, 2-hydroxyglutaric acid, dicarboxylic acids, and multiple acylglycines in urine, along with multiple acylcarnitines in plasma. Both died at a few days of life. Patient 1 showed compound heterozygosity for *ETFDH* mutations (c.50dup/p.His17GlnfsX6 in exon 2 and c.313A>G/p.Lys105Glu in exon 3). Patient 2 had a complete loss of ETF-DH activity and a homozygous mutation involving *ETFDH* (c.34+5G>C, donor splice site of intron 1). Patient 3 was the

sister of patient 2 and MADD diagnosis was obtained during pregnancy. The child died at 38 days of life of respiratory failure following aspiration pneumonia. Patient 4 made good progress under treatment (Table 1), yet primary MADD was confirmed by molecular genetic testing (homozygous known pathogenic mutations in *ETFA*: c.797C>T, p.Thr266Met, in exon 9). She is now 9 years old. Of note, levels of glutaric acid (1,669 vs 5,280–7,980 mmol/mol creatinine) and other markers in urine were lower for patient 4 than patients 1–3.

The remaining five patients with biochemical features of MADD were suspected of having secondary MADD.

Riboflavin deficiency linked to riboflavin deficiency in the mothers was suspected for three of these patients (referred to as 5–7). Their clinical presentations were comparable to neonatal MADD but biochemical abnormalities were milder. Notably, urine glutaric acid levels were between 175 and 2,272 mmol/mol creatinine (patients 5 and 6). For patients 5 and 6, vitamin supplementation completely normalized clinical and biochemical features. The patients did not have further episodes of decompensation under riboflavin supplementation and eventually without any therapy. Normalization of biochemical parameters and uneventful evolution after discontinuation of therapy are not consistent with the diagnosis of primary MADD. In addition, for patient 5, fatty acid oxidation flux analysis was normal in fibroblasts, and for patient 6, we did not detect mutations of *ETFA*, *ETFB*, or *ETFDH*. Patient 7 died at 3 days of life, and we suspect a secondary MADD deficiency, because only moderate abnormalities were detected for acylcarnitines and organic acids in plasma (urine analysis unavailable).

Our last two cases (patients 8 and 9) likely suffered from iatrogenic riboflavin deficiency. Both were premature newborns (32 and 31 weeks gestation, respectively), fed by parenteral nutrition for over 2 weeks. Clinical signs and metabolic acidosis appeared after 2 and 3 weeks, respectively. They showed milder but suggestive biochemical profiles of MADD. Patient 8 recovered upon multivitamin supplementation while patient 9 died before diagnosis.

All procedures were in accordance with the ethical standards of the local committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

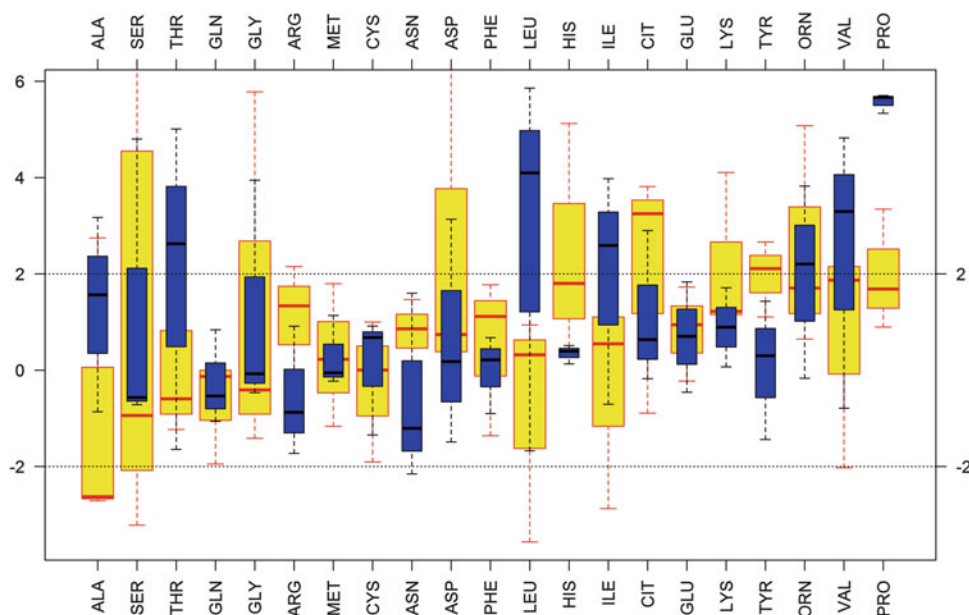
## Amino Acid Analyses and Statistics

Quantitative plasma amino acid analysis was performed by ion-exchange chromatography: 40- $\mu$ L deproteinized samples were injected into a JEOL AminoTac JLC-500/V

**Table 1** Summary of clinical and biochemical findings in patients with MADD or MADD-like presentation

Patient n/sex	Age of onset	Survival	Clinical picture	Glutaric acid (mmol/mol of creatinine) in urine	Other anomalies <sup>a</sup>	Proline/alanine (μmol/L) in plasma	Diagnosis
1/F	34 h	Deceased at 8 days	Hypoglycemia, feeding difficulties, hypotonia, mild hyperammonemia, hyperlactatemia, convulsion, hepatomegaly	7,080	Urine: EMA, IBG, BG, HG, 2-hydroxyglutaric acid, adipic acid, suberic acid	369/130	Primary MADD (ETF-DH deficiency)
2/M	1 h	Deceased at 4 days	Hypoglycemia, metabolic acidosis, mild hyperammonemia, lethargic coma, hypotonia, hepatomegaly, subependymal hemorrhage, PAH	6,507	Urine: EMA, 2-hydroxyglutaric acid, adipic acid, suberic acid, sebatic acid, HG, IBG, BG, IVG	622/126	Primary MADD (ETF-DH deficiency)
3/F	Antenatal diagnosis	Deceased at 38 days	Macrocephaly, dysmorphia, hepatomegaly, metabolic acidosis, hyperlactatemia, mild hyperammonemia, hypotonia	5,280	Urine: EMA, adipic acid, 2-hydroxyglutaric acid, IBG, IVG	NR/NR	Primary MADD (ETF-DH deficiency)
4/F	1 day	Alive	Feeding difficulties, hypotonia, hypothermia, hepatomegaly, hypoglycemia, hyperammonemia, hyperlactatemia, renal insufficiency	1,669	Urine: EMA, adipic acid, suberic acid, sebatic acid, HG, IBG, BG, IVG Plasma acylcarnitines: C4–C16:1	272/905	Primary MADD (ETFA deficiency)
5/F	12 h	Alive	Hypoglycemia, hyperlactatemia, pulmonary distress, metabolic acidosis, subependymal hemorrhage,	175	Urine: EMA, 2-hydroxyglutaric, IBG, IVG, HG, adipic acid, suberic acid, sebatic acid	1,152/1,134	Suspected secondary MADD
6/M	2 days	Alive after the neonatal period lost to follow-up	Hypoglycemia, hypotonia, feeding difficulties, hyperammonemia	2,272	Urine: adipic acid, suberic acid, sebatic acid, HG, SG, PG, BG, IVG	NR/NR	Suspected secondary MADD
7/F	1 day	Deceased at 3 days	Cardiorespiratory arrest, metabolic acidosis with hyperlactatemia, hepatomegaly, dilated left ventricle, PAH, liver dysfunction, pulmonary hemorrhage, multiorgan failure	2.87 <sup>b</sup>	Plasma: 2-hydroxyglutaric acid, EMA, adipic acid, suberic acid Plasma acylcarnitines: C16-OH, C14-DC, C18-DC, glutaryl/carnitine	1,457/429	Suspected secondary MADD
8/M	35 days	Alive	Metabolic acidosis, mild hyperlactatemia, pancytopenia, lethargy, hypotonia, myoclonia, hepatomegaly	535	Urine: EMA, 2-hydroxyglutaric, adipic acid, suberic acid, HG, SG, BG, IVG Plasma acylcarnitines: C3-C14	1,181/247	Inborn error of riboflavin deficiency
9/M	13 days	Deceased at 17 days	Abnormal movements, anemia, thrombopenia, and severe metabolic acidosis	1,018	Urine: EMA, 2-hydroxyglutaric, adipic acid, suberic acid, HG, SG, BG, IVG Plasma acylcarnitines: C4-C14	1,230/599	Inborn error of riboflavin deficiency
Control				<14		83–281/ 166–514	

EMA ethylmalonic, BG butyrylglycine, HG hexanoylglycine, IVG isovalerylglucose, IBG isobutyrylglycine, PG propionylglycine, IBG isobutyrylglycine, PAH pulmonary arterial hypertension, NR not reported  
<sup>a</sup> Increase of the reported metabolites  
<sup>b</sup> Plasma value in μmol/L (normal values 0.53 μmol/L)



**Fig. 1** Box plots of age-normalized levels of 21 plasma amino acids for samples from patients with primary (yellow) or presumably secondary (blue) MAD deficiency at presentation. Y-axis: age-normalized standard deviations from a hospital reference population.

Amino acids are ordered from the lowest (alanine, ALA) to the highest median levels (proline, PRO) across all samples. The boxes cover the  $\pm 25$ th percentile from the median (horizontal line in the boxes); the dotted lines cover the  $\pm 95$ th percentile from the median

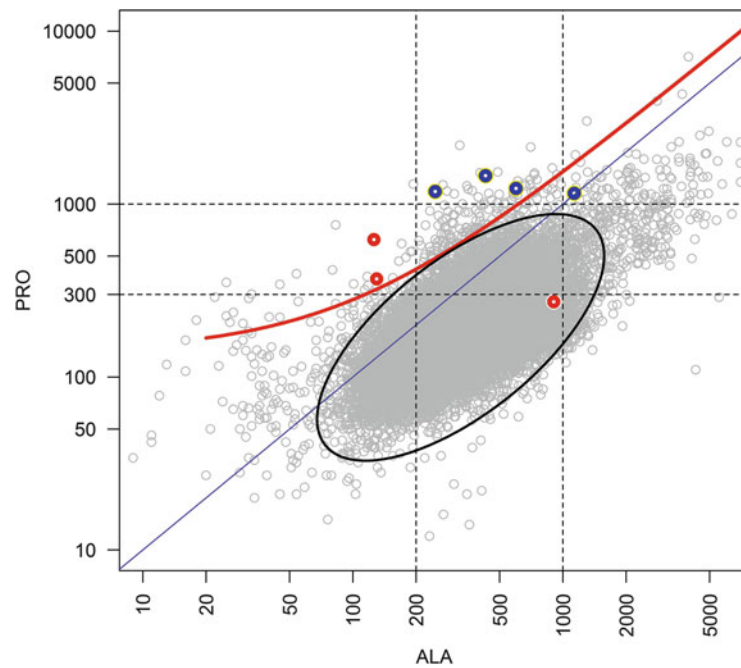
amino acid analyzer calibrated according to standards of known concentration. Amino acid identification was based on retention time and quantification was obtained by integration. Statistical analyses were performed in R (cran.r-project.org). In particular, the ellipse of Fig. 2 was obtained by robust covariance estimation (*covRob* module).

## Results

We first investigated individual amino acid profiles to assess the degree of hyperprolinemia relative to reference intervals. Of the seven cases tested for plasma amino acids, six patients showed hyperprolinemia (Table 1). Two of four cases of primary MADD had only mild hyperprolinemia, yet contrasting with a paradoxical, moderate decrease of alanine concentration below the normal/reference interval. The only primary MADD patient who survived (patient 4) never showed marked hyperprolinemia at diagnosis or during follow-up. All the informative cases suspected of secondary MADD (patients 5, 7, 8, 9) showed much more pronounced hyperprolinemia (1,152–1,457 vs 272–622  $\mu\text{mol/L}$ ), whereas alanine levels were only moderately increased. For patient 7, as the initial diagnostic workup did not include organic acid or acylcarnitine profiling, clinical presentation and hyperprolinemia suggested to complete biochemical investigations leading to the diagnosis.

To evaluate the relevance of the association between hyperprolinemia and biochemical features of MADD, we noticed that alanine showed the minimum median levels across all the patients compared to 20 additional amino acids (Fig. 1). Because of the observed dissociation between proline and alanine levels, we reasoned that the ratio or the difference between the levels of these two amino acids could be of diagnostic relevance. Figure 2 plots proline against alanine in our entire cohort of plasma amino acid profiles archived since 1995 including patients with known metabolic disorders ( $N = 53,338$ ). Figure 2 shows a clear correlation between alanine and proline, with on average higher concentrations of the former amino acid. We observed that some MADD-like profiles clearly deviate from this trend, characterized by high proline concentrations relative to alanine.

The plot shows that 3/4 tested patients with suspected secondary MADD were very unusual in having very high proline levels ( $>1,000 \mu\text{M}$ ) contrasting with lower alanine levels ( $<1,000 \mu\text{M}$ ). Two of three tested patients with primary MADD were also unusual in having moderate increases of proline ( $>300 \mu\text{M}$ ) contrasting with reduced alanine ( $<200 \mu\text{M}$ ). The plot also suggests that a potentially useful, continuous cutoff could be plasma proline at levels greater than  $1.4 \times (\text{alanine concentration}) + 140$  (red line in Fig. 2; in micromoles/liter). Only 153 of the 53,338 samples show proline values above such cutoff and would be regarded as consistent with MADD,



**Fig. 2** The plasma levels of alanine (“ALA”,  $x$ -axis) and proline (“PRO”,  $y$ -axis) in micromoles/liter are shown for 53,338 samples corresponding to our complete cohort (gray). The samples collected at presentation from the patients reported in this study are shown as red (newborns with primary MADD) or blue circles (newborn with suspected secondary MADD). The equality line is blue, indicating that

average reference alanine levels are greater than proline. Useful cutoffs are shown by black dashed lines and a proposed continuous cutoff by a red line (corresponding to the equation  $PRO = 1.4 \times ALA + 140$ ) (see text). The black ellipse represents the estimated bivariate normal distribution at  $\alpha = 10^{-5}$

with a  $p$ -value  $< 0.003$  (the same  $p$ -value is obtained by restricting the analysis to the first sample of each patient in the cohort, which yields 35 positive out of 34,525 patients). This cutoff is a tangent to the ellipse of Fig. 2, corresponding to a bivariate reference distribution, at alpha error of  $10^{-5}$ , thus supporting the conclusion that the values associated with most neonatal MADD or MADD-like samples are highly deviant.

These analyses showed an overrepresentation of MADD-like cases among patients with hyperprolinemia, confirming the relevance of the association between these biochemical features. In addition, we suggest that hyperprolinemia with dissociated alanine levels can be useful to suggest a MADD-like disorder. Of note, other etiologies with proline much greater than alanine were primary hyperprolinemia, E3 (DLD) deficiency, lysinuric protein intolerance, and peri-mortem conditions.

## Discussion

Early reports pointed to hyperprolinemia as a common feature of MADD, yet plasma proline is currently not regarded as a marker of the disease. Our study indicates that it is a frequent finding both in primary and in suspected cases of secondary early-onset forms of MADD. Our

uniquely large cohort of  $> 50,000$  plasma amino acid samples also indicates that the simple dissociation between high proline and lower alanine levels may be suggestive of the diagnosis, by using simple cutoffs such as proline levels greater than 140% of alanine levels plus  $140 \mu\text{M}$ . This is of particular interest considering that secondary MADD readily responds to riboflavin supplementation, provided that the diagnosis is suggested promptly. In addition, a small fraction of primary MADD cases can also respond to therapy involving riboflavin, carnitine, and appropriate diet.

Proline dehydrogenase (or proline oxidase (POX)) is a flavoprotein, well characterized in microorganisms and highly conserved throughout eukaryotes and bacteria (Servet et al. 2012; Tanner 2008). POX oxidizes proline to produce  $\Delta^1$ -pyrroline-5-carboxylate (P5C), the first step of proline catabolism. Reduced POX may then give electrons to the electron transfer chain directly via the ubiquinone pool (Moxley et al. 2011), thus without requiring ETF (Wanduragala et al. 2010). Gene sequence comparison and absorption spectral characterization of its catalytic site have suggested that human POX features are comparable to those in microorganisms (Tallarita et al. 2012; Tanner 2008). Because POX is a flavoprotein, it is reasonable to propose that hyperprolinemia observed in riboflavin deficiency may be secondary to depletion in the FAD cofactor. The reason why hyperprolinemia is observed

in MADD is still unclear. Secondary coenzyme Q10 deficiency has been observed in riboflavin-responsive MADD (Cornelius et al. 2013). It is tempting to speculate that MADD might induce alterations of the ubiquinone pool or of riboflavin metabolism leading to hyperprolinemia.

Plasma proline was most prominently elevated in the suspected cases of secondary MADD at the time of presentation and was also found in a patient with Brown–Vialletto–Van Laere syndrome during a period of cardiorespiratory distress that preceded death (data not shown). Therefore, we suggest that hyperprolinemia may be a marker of acute decompensation. Nevertheless, it was not found at any time point in a primary MADD case that survived, and the other patients with primary MADD had absolute levels of proline that were not very elevated, except if compared to alanine.

Furthermore, proline levels were higher in secondary MADD, offering the opportunity to quickly refine MADD diagnoses.

We cannot completely rule out differential diagnoses for hyperprolinemia such as medical treatment containing proline, parenteral nutrition, or gelatin administration (Illsinger et al. 2006). However, the degree of hyperprolinemia relative to other amino acids such as alanine, and its association with biochemical features of MADD in different contexts, times, and geographical areas, is not really consistent with this association being coincidental. Hyperlactatemia also induces hyperprolinemia by inhibition of proline oxidase (Kowaloff et al. 1977). However, alanine and proline are both known to increase concomitantly with increased lactate concentrations, yet alanine did not increase as much as proline or actually decreased in some cases (Fig. 2). Hyperprolinemia can also be observed in peri-mortem profiles, characterized by generalized hyperaminoacidemia with very low arginine concentrations, which was clearly different from our profiles.

Our study highlighted the clinical heterogeneity associated with biochemical profiles of MADD. A significant part of suspected neonatal MADD may be secondary forms, treatable with riboflavin. For populations at risk of riboflavin deficiency like preterm newborns under parenteral nutrition, riboflavin supplementation should be considered with particular attention. Critical care recommendations may include riboflavin supplementation as part of a multivitamin cocktail as a first-line therapy for severely ill newborns. Based on our data, in some areas, education of neonatologists may be required to ensure complete prevention of iatrogenic or other causes of secondary MADD.

As shown by our study based on >50,000 plasma amino acid profiles, biochemical profiles of MADD were overrepresented among cases of hyperprolinemia with simple cutoffs ( $p < 0.003$ ), and other diagnoses with similar

alanine and proline levels are associated with very different clinical pictures (see above). This observation is particularly relevant for countries like France where plasma amino acid profiles are ordered before acylcarnitines and urine organic acids profiles. Of particular interest, proline concentrations were more pronounced in secondary MADD that is treatable but can lead to death if untreated. Hyperprolinemia may be a first-line indication of the diagnosis prompting the introduction of MADD therapy.

Our study suggests that the absolute difference between proline to alanine levels in plasma may be a good marker of MAD deficiency, in particular for secondary etiologies, and could contribute to a more effective management of this disorder.

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## Synopsis

Hyperprolinemia and more precisely high proline/alanine ratio may be a good marker of MAD deficiency.

## Compliance with Ethics Guidelines

### Conflict of Interest

Clément Pontoizeau, Florence Habarou, Anaïs Brassier, Alice Veauville-Merllié, Coraline Grisel, Jean-Baptiste Arnoux, Christine Vianey-Saban, Robert Barouki, Bernadette Chadefaux-Vekemans, Cécile Acquaviva, Pascale de Lonlay, and Chris Ottolenghi declare that they have no conflict of interest.

## Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

## Details of the Contributions of Individual Authors

AB, CG, J-BA, and PdL collected the data. CP, FH, AVM, CVS, CA, PdL, and CO analyzed the data. CP, FH, CVS,

CA, PdL, and CO wrote the manuscript. All authors read and approved the final manuscript.

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