The Relationship Between Circulating Neuregulin-1 and Coronary Collateral Circulation in Patients with Coronary Artery Disease

Maozhi Huang,^{1*} MM, Jianping Zheng,^{1*} MM, Ziguo Chen,¹ MB,
Chaoqun You,¹ MM and Qilei Huang,¹ MM

Summary

Coronary collateral circulation (CCC) plays a crucial role in myocardial blood supply, especially for ischemic myocardium. Previous study has shown that neuregulin-1 is a prominent angiogenic factor in diabetic cardiomyopathy, whereas the relationship between neuregulin-1 and CCC has not been investigated. Thus, we aimed to investigate relationship between circulating neuregulin-1 levels and CCC in stable coronary artery disease patients.

Coronary artery disease patients with a stenosis of $\geq 90\%$ as evidenced by coronary angiography were included in our study. According to the Rentrop-Cohen classification, coronary collateral degree was graded as 1 to 4. Patients with collateral degree grade 0 or 1 were enrolled in poor CCC group, whereas patients with grade 2 or 3 were enrolled in good CCC group.

Plasma neuregulin-1 level was significantly increased in good collateral group and positively related to Rentrop grade ($P < 0.01$). Multivariate regression analysis and ROC (receiver operating characteristic curve) revealed that plasma neuregulin-1 could predict CCC status effectively.

Increased plasma neuregulin-1 level was related to better CCC in patients with coronary artery disease. Neuregulin-1 was an independent and reliable predictor for good coronary collateral development and provided a potential therapeutic strategy to reduce myocardial ischemia injury.

(Int Heart J 2020; 61: 115-120)

Key words: Myocardial ischemia, Biomarker, Angiogenesis

Coronary collateral circulation (CCC) is crucial and prognostically relevant in patients with coronary artery disease (CAD), which serves as a natural bypass to supply essential blood to the ischemic myocardium.¹⁾ Well-developed coronary collateral arteries have been evidenced previously to limit infarct size, reduce arrhythmia, preserve cardiac function, and eventually improve cardiovascular prognosis.²⁻⁴⁾ Coronary collaterals derive embryonic period and develop throughout the whole life, but the interindividual variation of coronary collateral coverage density and function is remarkably wide.⁵⁾ The understanding of coronary development and its mechanism is beneficial to provide novel therapy to coronary ischemic disease. Until now, no noninvasive technique is available for collateral function assessment.^{5,6)} Therefore, the identification of a circulating biomarker to discriminate good or poor CCC and the exploration of its underlying mechanism would be of great clinical significance.

Neuregulin-1 (NRG-1) is a paracrine factor belonging to the epidermal growth factor family which is identified to have a significant role in CAD.^{7,8)} It is essential for the

cardiovascular system development and repair under various stress.^{9,10)} Recently, increasing evidence has revealed that NRG-1 was closely related to angiogenesis^{11,12)}. NRG-1 can be produced by vascular endocardial and cardiac microvascular endothelium and is a positive regulator of angiogenesis.¹³⁾ In addition, NRG-1 has been shown to promote myocardial angiogenesis in the ischemic and diabetic myocardium and is involved in the vascular endothelial growth factor (VEGF) / Angiopoietin-1(Ang-1) angiogenesis signaling events.^{11,14,15)} These findings revealed that NRG-1 might participate in the development of CCC and could function as a potential biomarker. However, the association between NRG-1 and CCC has not been explored in CAD patients.

In this study, we tested the relationship between circulating NRG-1 level and CCC status and its predictive power as a CCC biomarker. Besides, the association of NRG-1 with VEGF and Ang-1 was detected to explore the underlying mechanism.

From the ¹Department of Cardiology, The First Hospital of Nanping City, Fujian Medical University, Fujian, China.

* These authors contributed equally to the work.

Address for correspondence: Qilei Huang, MM, Department of Cardiology, The First Hospital of Nanping City, Fujian Medical University, 317 Zhongshan Road, Nanping City, Fujian Province, 353000, China. E-mail: qilei_huang@protonmail.com

Received for publication May 30, 2019. Revised and accepted October 3, 2019.

Released in advance online on J-STAGE January 17, 2020.

doi: 10.1536/ihj.19-277

All rights reserved by the International Heart Journal Association.

Methods

Study population: Patients who received coronary angiography from 2016 to 2018 at our hospital with at least one coronary artery > 90% degree of diameter stenosis were enrolled. The Rentrop-Cohen classification was used to grade collateral degree.¹⁶⁾ Grades 2 and 3 were considered as good CCC, whereas grades 0 and 1 were considered as poor CCC. Exclusion criteria included coronary artery lumen stenosis < 90%, recent acute coronary syndrome (< 6 months), heart failure, cardiomyopathy, valvular heart disease, previous revascularization history, peripheral vascular disease, chronic obstructive pulmonary disease, chronic kidney or liver disease, previous diagnosis of malignancy, and concomitant inflammatory diseases. A detailed physical examination and laboratory analysis were conducted on all patients. The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of our hospital. All patients were informed, and consent was obtained.

Coronary collateral scoring: Coronary angiography was performed in the standard Judkins manner. The CCC scoring was based on the Rentrop-Cohen method: 0 = no visible filling of collateral vessels; 1 = collateral filling of branches of the vessel without visualization of epicardial segment; 2 = partial collateral filling of the epicardial segment; 3 = complete collateral filling of the epicardial segment. Sixty-five patients with grade 0 or 1 collateral vessels were in poor collateral circulation group; 63 patients with grade 2 or 3 collateral vessels were included in good collateral circulation group.

Detection of circulating NRG-1 through ELISA: Blood samples from all patients were collected in EDTA vacuum tubes and then 3,000 rpm centrifuged for 10 minutes at room temperature. The plasma was separated and stored in aliquots. Circulating NRG-1 was detected using ELISA (R&D cat# DY377) according to the manufacturer's instructions. Briefly, the standards and diluted samples were added to 96-well plate coated with the capture antibody. By serial washing, detection antibody and streptavidin-HRP were sequentially added and incubated. After adding substrate solution protected from light for 20 minutes, the stop solution was finally added, and the absorbance was measured at 450 nm. The detection limit level of circulating NRG ranged from 0.3 to 30 ng/mL.

Detection of plasma Ang-1 and VEGF levels: Human ELISA Kit from R&D Systems was used to assay plasma Ang-1 and VEGF-A according to the manufacturer's instructions. In brief, 100 μ L of sample was incubated with biotin conjugate anti-Ang-1 or anti-VEGF-A antibody as a primary antibody. After serial washing, streptavidin-HRP was added and then incubated for 1 hour. The absorbance was read at 450 nm.

Statistical analysis: SPSS 19.0 (SPSS Inc., Chicago, Illinois) was used for statistical analysis. Continuous variables were expressed as mean $\pm$ standard deviation, and categorical variables were defined as percentages. Continuous variables were compared using Student's *t*-test or Mann-Whitney *U* test. Categorical data were compared using a chi-squared test. Differences among different groups were assessed using one-way ANOVA compar-

isons. The association between the independent variables and CCC was assessed using univariate and multivariate logistic regression analyses. To determine the NRG-1 optimal cut-off value in discrimination of coronary collateral development, the receiver operating characteristic (ROC) curve analysis was performed. A *P* value of < 0.05 was considered to be statistically significant.

Results

Baseline characteristics: Clinical characteristics and biochemical parameters of the 128 patients grouped by collateral development were listed in Table I. There were 65 patients with poor CCC and 63 with good CCC. Most patients of both groups were male, and the mean age was 61.5 ± 6.5 in good CCC group and 62.8 ± 8.9 in poor CCC group. There were no significant differences in age; gender; BMI; family history of CAD, diabetes mellitus, and hypertension; left ventricular ejection fraction, NT-proBNP, creatinine, and uric acid. Lipid profiles, including TC, TG, LDL-C, and HDL-C, and medication usage were not different between the two groups. The ratio of patients with active smoking was higher in good CCC group. Hs-CRP level was significantly increased in poor CCC than good CCC. The findings of coronary angiography were presented in Table II. No difference in number of diseased vessels was found between the two groups. However, the frequency of occluded right coronary artery was higher in good CCC group than in poor CCC group (58.3% versus 35.2%, *P* < 0.05).

Results of plasma NRG-1 detection: Plasma level of NRG-1 was significantly higher in good CCC group than in poor CCC group (*P* < 0.05) (Figure 1). When NRG-1 level was compared based on Rentrop scoring, the lowest level of NRG-1 was found in Rentrop 0 group, whereas the highest level was found in Rentrop 3 group (*P* < 0.05) (Figure 1). It was reported previously that NRG-1 was associated with VEGF and Ang-1. Therefore, plasma VEGF and Ang-1 levels and their correlations with NRG-1 were detected. The results revealed that the plasma levels of VEGF and Ang-1 were significantly increased in good CCC group than in poor CCC group. Besides, there was a positive correlation of plasma NRG-1 with VEGF (*r* = 0.834, *P* < 0.05) and Ang-1 (*r* = 0.612, *P* < 0.05) (Figure 2). We next performed univariate and multivariate logistic regression analyses to determine the independent predictors of collateral development. Our univariate analysis results revealed that smoking, hs-CRP, VEGF, Ang-1, NRG-1, and occluded RCA were possible confounding factors for good coronary collateral formation, whereas NRG-1 and occluded RCA still remained as independent predictors in the multivariate regression analysis results (Table III). Additionally, ROC analysis revealed that 7.75 ng/mL NRG-1 level generated 80.95% sensitivity and 73.85% specificity for the prediction of good coronary collateral formation (Figure 3).

Discussion

In this study, the association between NRG-1 and CCC was assessed. The main findings were as follows:1)

Table I. Clinical Characteristics and Biochemical Parameters of the Patients

Variable	Good CCC (n = 63)	Poor CCC (n = 65)	P
Age (years)	61.5 ± 6.5	62.8 ± 8.9	NS
Gender (Male)	69%	71%	NS
BMI (kg/m ²)	27.1 ± 1.9	26.8 ± 2.3	NS
Hypertension	68%	70%	NS
Diabetes mellitus	29%	31%	NS
Smoking	48%	22%	< 0.05
TG (mmol/L)	1.6 ± 0.9	1.5 ± 0.3	NS
TC (mmol/L)	4.6 ± 0.5	4.5 ± 1.0	NS
HDL-C (mmol/L)	1.1 ± 0.3	1.1 ± 0.5	NS
LDL-C (mmol/L)	2.9 ± 0.9	2.8 ± 0.6	NS
Creatinine (mg/dL)	0.8 ± 0.3	0.9 ± 0.4	NS
Uric acid (mg/dL)	5.5 ± 1.5	5.7 ± 1.2	NS
hs-CRP (mg/L)	3.4 ± 0.9	5.1 ± 1.2	< 0.05
LVEF	58 ± 6.5	59 ± 7.8	NS
NT-proBNP (pg/mL)	68 ± 3.2	69 ± 4.1	NS
Family history of CAD	25%	27%	NS
B-blockers	69%	71%	NS
ACEIs	55%	52%	NS
Statins	29%	33%	NS
ARBs	12%	14%	NS
CCBs	19%	21%	NS
Aspirin	71%	73%	NS

BMI indicates body mass index; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; NT-proBNP, N-terminal brain natriuretic peptide; ACEIs, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; CCBs, calcium channel blockers; and NS, no significance.

Table II. Coronary Angiographic Findings of the Patients

Variable	Good CCC	Poor CCC	P
Occluded LAD	49.2%	60.4%	NS
Occluded LCX	18.6%	23.7%	NS
Occluded RCA	58.3%	35.2%	< 0.05
One-vessel disease	32.5%	33.6%	NS
Two-vessel disease	48.5%	44.3%	NS
Three-vessel disease	19.0%	22.1%	NS

LAD indicates left anterior descending artery; LCX, left circumflex artery; and RCA, right coronary artery.

NRG-1 plasma level was elevated in patients with good CCC than poor CCC; 2) the circulating NRG-1 level was an independent predictor for good CCC development; 3) plasma NRG-1 was found positively and significantly correlated with VEGF and Ang-1, which indicated a possible mechanical link in coronary collateral development under ischemic insult.

CAD is the leading cause of death worldwide. Although coronary revascularization, such as percutaneous coronary intervention (PCI) therapy and coronary artery bypass grafting, is an efficient and mature therapeutic method, not every patient was suitable for this therapy. Besides, certain downsides of revascularization therapy could not be overlooked, including the limited vessel treatment segments, restenosis after PCI, and various post-operative complications. Increasing evidence revealed that well-developed CCC not only provided a potential source of blood supply to the myocardium but also functionally relieved ischemic symptoms and improved prognosis. The degree of collateralization varies considerably among pa-

tients. Many determinants, including genetic background, physical exercise, duration of clinical symptoms, coronary artery stenosis degree, hypertension, and diabetes mellitus, have been proposed to be associated with CCC development.¹⁷⁻²⁰⁾ However, the underlying mechanism has not been completely understood yet. In addition, the current method to assess collateral circulation is invasive, which limited the widespread assessment of CCC among patients.

NRG-1 is a member of the epidermal growth factor family. Previous studies have proven that NRG-1 is essential for cardiac homeostasis and repair.²¹⁾ Recently, mounting evidences revealed that NRG-1 is a crucial regulator of angiogenesis. It has been reported that NRG-1 can induce endothelial angiogenesis *in vitro* and increase the number of micro-vessels formed in the ischemic myocardium.^{22,23)} In diabetic cardiomyopathy, NRG-1 treatment can significantly promote myocardial angiogenesis and preserve cardiac function.¹¹⁾ The mechanisms of the positive angiogenesis role for NRG-1 can be partly attributed to the elevated expression of VEGF and Ang-1.¹¹⁾ However, another study has revealed that the expression and release of NRG-1 were significantly increased in human cardiac microvascular endothelial cells under VEGF and Ang-1 stimulation, which suggested that these angiogenic factors could regulate NRG-1 signaling pathway.¹⁴⁾ Although there is no conclusion of the precious casual association among VEGF, Ang-1, and NRG-1, we can speculate that there may be a positive feedback among VEGF/Ang-1-NRG-1 signaling pathways. Consistent with these findings, our results have demonstrated that NRG-1, VEGF, and Ang-1 were all significantly increased in good

Figure 1. A: NRG-1 levels in poor and good coronary collateral circulation groups. B: NRG-1 levels according to Rentrop groups.

Figure 2. A: VEGF levels in poor and good coronary collateral circulation groups; B: Ang-1 levels in poor and good coronary collateral circulation groups; C: Correlation between the level of VEGF and NRG-1; D: Correlation between the level of Ang-1 and NRG-1.

CCC group, and there was a positive correlation among them.

Previous clinical study has revealed that elevated circulating levels of NRG-1 are related to stress-mediated ischemia and inversely associated with CAD severity in stable patients.²⁴⁾ In addition, NRG-1 level is independently related to heart failure severity and adverse out-

comes.²⁵⁾ Furthermore, it has been shown that NRG-1 has a great potential in identifying breast cancer patients who have a higher risk of heart failure under cardiotoxic medications.²⁶⁾ These results highlighted that NRG-1 can be a biomarker representing underlying cardiac stress and repair. Therefore, increased levels of NRG-1 may suggest that activated endogenous cardiac compensatory efforts re-

Table III. Univariate and Multivariate Analysis for Good Coronary Collateral Development

Variable	Univariate regression analysis		Multivariate regression analysis	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
Smoking (%)	2.1 (1.1-4.7)	< 0.05	1.2 (0.3-5.5)	NS
hs-CRP (mg/L)	0.3 (0.1-0.8)	< 0.05	0.9 (0.4-2.5)	NS
VEGF	2.56 (1.2-7.3)	< 0.05	3.1 (0.1-52.3)	NS
Ang-1	1.58 (1.3-3.2)	< 0.05	1.2 (0.3-1.5)	NS
NRG-1	5.8 (2.5-13.2)	< 0.05	7.1 (1.4-30.2)	< 0.05
Occluded RCA	3.5 (1.3-7.5)	< 0.05	1.8 (1.4-5.9)	< 0.05

hs-CRP indicates high-sensitivity C-reactive protein; RCA, right coronary artery; and NS, no significance.

Figure 3. Receiver operating characteristic curve analysis of NRG-1 for the prediction of good coronary collateral circulation.

spond to various stress. Because of the cardioprotective role of NRG-1, recombinant human NRG-1 has been tested in clinical trials, which displayed a promising result of improving cardiac function and prognosis.^{27,28} In this study, we found that NRG-1, VEGF, and Ang-1 were significantly increased in good CCC group than in poor CCC group. Furthermore, we postulated that circulating NRG-1 not only is a symbol of cardiac stress but also represents effective cardiac repair and adoption, which might be activated *via* NRG-1 or VEGF/Ang-1 signaling. Our findings have shown that the presence of occluded RCA and active smoking were more frequent in good CCC group than in poor CCC group, whereas hs-CRP was reduced in good CCC, which was consistent with previous studies.²⁹⁻³² Although these variables were meaningful confounding factors in univariate regression analysis, only NRG-1 and occluded RCA still remained as independent predictors after multivariate logistic regression analysis. Therefore, inflammation, segments and degree of vascular occlusion, and active smoking effects may also be contributors of the stimulation of coronary collateralization, which still needs more basic research in large cohort.

The main limitation of our study is its relatively small population. Besides, Rentrop score was used to assess CCC instead of collateral flow index, which was more accurate and informative.

Conclusion

In conclusion, our study revealed that the circulating NRG-1 was an independent and reliable predictor of good CCC development, probably *via* the direct effects of

NRG-1 or the increased VEGF/Ang-1 signaling. These results may pave the way to the development of a novel strategy to reverse the myocardial ischemic impairment of patients who are not appropriate for current revascularization treatment.

Disclosure

Conflicts of interest: No potential conflicts of interest were disclosed.

References

1. Meier P, Schirmer SH, Lansky AJ, Timmis A, Pitt B, Seiler C. The collateral circulation of the heart. *BMC Med* 2013; 11: 143.
2. Smith RD, Ilesley CD. Clinical contribution of the collateral circulation to myocardial protection. *Coron Artery Dis* 2004; 15: 393-8.
3. Berry C, Balachandran KP, L'Allier PL, Lespérance J, Bonan R, Oldroyd KG. Importance of collateral circulation in coronary heart disease. *Eur Heart J* 2007; 28: 278-91.
4. Gungor H, Eryilmaz U, Akgullu C, *et al.* Preoperative poor coronary collateral circulation can predict the development of atrial fibrillation after coronary artery bypass graft surgery. *Coron Artery Dis* 2013; 24: 572-6.
5. Zimarino M, D'Andreanmatteo M, Waksman R, Epstein SE, De Caterina R. The dynamics of the coronary collateral circulation. *Nat Rev Cardiol* 2014; 11: 191-7.
6. Kurtul A, Ozturk S. Prognostic value of coronary collaterals in patients with acute coronary syndromes. *Coron Artery Dis* 2017; 28: 406-12.
7. Tian QP, Liu ML, Tang CS, Xue L, Pang YZ, Qi YF. Association of circulating Neuregulin-4 with presence and severity of coronary artery disease. *Int Heart J* 2019; 60: 45-9.
8. Sato T, Minatsuki S. Neuregulin-4, an adipokine, as a residual risk factor of atherosclerotic coronary artery disease. *Int Heart J* 2019; 60: 1-3.
9. Mendes-Ferreira P, De Keulenaer GW, Leite-Moreira AF, Brás-Silva C. Therapeutic potential of neuregulin-1 in cardiovascular disease. *Drug Discov Today* 2013; 18: 836-42.
10. Wang X, Liu Z, Duan HN, Wang L. Therapeutic potential of neuregulin in cardiovascular system: can we ignore the effects of neuregulin on electrophysiology? *Mini Rev Med Chem* 2016; 16: 867-71.
11. Gui C, Zeng ZY, Chen Q, Luo YW, Li L, Chen LL. Neuregulin-1 promotes myocardial angiogenesis in the rat model of diabetic cardiomyopathy. *Cell Physiol Biochem* 2018; 46: 2325-34.
12. Hedhli N, Kalinowski A, S Russell K. Cardiovascular effects of neuregulin-1/ErbB signaling: role in vascular signaling and angiogenesis. *Curr Pharm Des* 2014; 20: 4899-905.
13. Lemmens K, Segers VF, Demolder M, De Keulenaer GW. Role of neuregulin-1/ErbB2 signaling in endothelium-cardiomyocyte

- cross-talk. *J Biol Chem* 2006; 281: 19469-77.
14. Wu C, Gui C, Li L, Pang Y, Tang Z, Wei J. Expression and secretion of neuregulin-1 in cardiac microvascular endothelial cells treated with angiogenic factors. *Exp Ther Med* 2018; 15: 3577-81.
 15. Hedhli N, Dobrucki LW, Kalinowski A, *et al.* Endothelial-derived neuregulin is an important mediator of ischaemia-induced angiogenesis and arteriogenesis. *Cardiovasc Res* 2012; 93: 516-24.
 16. Rentrop KP, Cohen M, Blanke H, Phillips RA. Changes in collateral channel filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. *J Am Coll Cardiol* 1985; 5: 587-92.
 17. de Marchi SF. Determinants of human coronary collaterals. *Curr Cardiol Rev* 2014; 10: 24-8.
 18. Seiler C, Stoller M, Pitt B, Meier P. The human coronary collateral circulation: development and clinical importance. *Eur Heart J* 2013; 34: 2674-82.
 19. Möbius-Winkler S, Uhlemann M, Adams V, *et al.* Coronary collateral growth induced by physical exercise: results of the impact of intensive exercise training on coronary collateral circulation in patients with stable coronary artery disease (EXCITE) trial. *Circulation* 2016; 133: 1438-48; discussion 1448.
 20. Stoller M, Seiler C. Salient features of the coronary collateral circulation and its clinical relevance. *Swiss Med Wkly* 2015; 145: w14154.
 21. Odiete O, Hill MF, Sawyer DB. Neuregulin in cardiovascular development and disease. *Circ Res* 2012; 111: 1376-85.
 22. Russell KS, Stern DF, Polverini PJ, Bender JR. Neuregulin activation of ErbB receptors in vascular endothelium leads to angiogenesis. *Am J Physiol* 1999; 277: H2205-11.
 23. Xiao J, Li B, Zheng Z, *et al.* Therapeutic effects of neuregulin-1 gene transduction in rats with myocardial infarction. *Coron Artery Dis* 2012; 23: 460-8.
 24. Geisberg CA, Wang G, Safa RN, *et al.* Circulating neuregulin-1 beta levels vary according to the angiographic severity of coronary artery disease and ischemia. *Coron Artery Dis* 2011; 22: 577-82.
 25. Ky B, Kimmel SE, Safa RN, *et al.* Neuregulin-1 beta is associated with disease severity and adverse outcomes in chronic heart failure. *Circulation* 2009; 120: 310-7.
 26. Geisberg CA, Abdallah WM, da Silva M, *et al.* Circulating neuregulin during the transition from stage A to stage B/C heart failure in a breast cancer cohort. *J Card Fail* 2013; 19: 10-5.
 27. Gao R, Zhang J, Cheng L, *et al.* A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. *J Am Coll Cardiol* 2010; 55: 1907-14.
 28. Jabbour A, Hayward CS, Keogh AM, *et al.* Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *Eur J Heart Fail* 2011; 13: 83-92.
 29. Söğüt E, Kadi H, Karayakalı M, Mertoglu C. The association of plasma vitamin A and E levels with coronary collateral circulation. *Atherosclerosis* 2015; 239: 547-51.
 30. Koerselman J, de Jaegere PP, Verhaar MC, Grobbee DE, van der Graaf Y, SMART Study Group. Coronary collateral circulation: the effects of smoking and alcohol. *Atherosclerosis* 2007; 191: 191-8.
 31. Zhou JP, Tong XY, Zhu LP, *et al.* Plasma Omentin-1 level as a predictor of good coronary collateral circulation. *J Atheroscler Thromb* 2017; 24: 940-8.
 32. Fan Y, Li S, Li XL, *et al.* C-reactive protein as a predictor for poor collateral circulation in patients with chronic stable coronary heart disease. *Ann Med* 2016; 48: 83-8.