

BREAST CANCER AND THE STROMAL FACTOR.
THE "PROMETASTATIC HEALING PROCESS" HYPOTHESIS

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Abstract The correlation between axillary status and several histological features of breast carcinomas has been well established, however stromal changes have rarely been analyzed. Detailed clinicopathological review of 1803 patients with infiltrating breast carcinoma was performed. Stromal myxoid changes (SMC), size (T2-T3: > 2 cm, T1c: 1-2 cm, T1 a-b: < 1cm), fibrotic focus, age, lymphovascular embolizations, tumor infiltrating lymphocytes (TIL), multifocality, histological grade (G), estrogen receptors (ER), progesterone receptors (PR) and HER2 were semi-quantitated in two or three grades and correlated to axillary status. SMC3 followed by T2-T3, G3, fibrotic focus, T1c, embolizations, SMC2, TIL2, G2 and multifocality were strongly associated with positive axillary nodes; an inverse association was found with ER+++ and PR+++ . Our findings support a critical role of the peritumoral stroma in the development of metastases. These stromal alterations should be remarked in routine pathology reports as they can be easily assessed and provide important information about tumor biology and aggressiveness. They could also become, in a future, the target of novel therapeutics.

Key words: breast cancer, stroma, metastases, hyaluronan

Resumen *El factor estromal en el cáncer de mama. La hipótesis del "proceso cicatrizal prometastático".*

La correlación entre estado axilar y varias características histológicas de los carcinomas de mama está bien establecida, sin embargo los cambios estromales rara vez fueron analizados. En el presente trabajo se realizó una revisión detallada de las características clínico-patológicas de 1803 pacientes con carcinoma infiltrante de mama. Los cambios mixoides estromales (SMC), el tamaño tumoral (T2-T3: > 2 cm, T1c: 1-2 cm, T1 a-b: < 1 cm), el foco fibroso, la edad, embolizaciones tumorales linfovascuales, infiltración linfocitaria tumoral (TIL), multifocalidad, grado histológico (G), los receptores estrogénicos (RE) y los receptores progestacionales (RP) y HER2 fueron semicuantificados en dos o tres grados y correlacionados con el estado axilar. El estudio multivariante demostró la asociación entre SMC grado 3 seguido por el tamaño tumoral T2-T3, G3, foco fibroso, T1c, embolizaciones tumorales linfovascuales, SMC2, TIL2, G2, multifocalidad y presencia de ganglios axilares metastásicos ($p < 0.0001$). Asimismo pudo comprobarse una asociación inversa entre RE+++ y RP+++ ($p < 0.0001$) con la presencia de metástasis axilares. Nuestros hallazgos sugieren un rol crítico del estroma peritumoral en el desarrollo de metástasis. Estas alteraciones estromales deberían, en nuestra opinión, constar en los informes de patología quirúrgica dado que son de fácil evaluación y aportan importante información acerca de la biología y agresividad tumoral. Además podrían convertirse, en un futuro, en el blanco de nuevas terapéuticas.

Palabras clave: cáncer de mama, estroma, metástasis, ácido hialurónico

When only conventional histopathology is utilized for the assessment of breast cancer, clinicians rely on a few factors: histological grade (G), size and nodal status. They add as secondary predictive factors the hormone receptors expression and the amplification of the oncogene HER2. The most important of these, the presence of metastatic axillary lymph nodes (MALN), is a significant predictor of survival in patients with infiltrative breast carcinoma¹.

Tumor size is a well recognized pathological feature that predicts MALN, however it is not uncommon to find small tumors with positive nodes and large tumors without metastases, a fact that suggests other factors besides tumor size are involved.

Carcinomas show two discrete but interdependent compartments: the malignant cells and the stroma they induce within which they are dispersed. The latter usually suffers three essential histological modifications that can be easily assessed by a surgical pathologist: stromal myxoid changes (SMC), tumor infiltrating lymphocytes (TIL) and desmoplasia.

SMC are a provisional myxoid-type reactive matrix composed of material that stains for Alcian Blue and is

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found interspersed between collagen fibers. Hyaluronan (HA) is one of the major components of this myxoid matrix and its synthesis is stimulated by the interaction between malignant cells and stromal fibroblasts². Auvinen et al³ found a relationship between HA, poorly differentiated tumor cells and axillary nodes positivity. Other authors have reported that HA is a fundamental piece of the metastatic process⁴⁻⁶. Also, a myxoid stroma was associated with advanced rectal cancer⁷, microinvasive adenocarcinoma of the endometrium⁸ and aggressive tumor behavior in vulvar squamous cell carcinomas⁹.

At sites of tumor invasion, TIL follow the SMC and these inflammatory cells, traditionally considered a good histopathological prognostic factor, have a paradoxical effect, as reviewed recently¹⁰. In addition, pronounced desmoplasia, called by Hasebe fibrotic focus, was found to be associated with cancer aggressiveness¹¹.

With emphasis on these components we have investigated several clinicopathological features of breast carcinomas, their interactions and their association with MALN to study the role of the peritumoral stroma in the development of metastases.

Materials and Methods

A total of 2787 consecutive cases of breast cancer surgical specimens, from the period between January, 1987 and July, 2006, were retrieved from the files of the Department of Pathology of the Italian Hospital of Buenos Aires. Cases without axillary sampling (axillary dissection or sentinel lymph node biopsy) or with either preoperative radiotherapy or neoadjuvant chemotherapy were not included because of the epithelial and stromal distortion caused by treatments, leaving a total of 1803 cases for evaluation. All tumors studied had been formalin fixed. Four micron-thick hematoxylin and eosin-stained sections were examined in all cases.

The histological slides were approached with a double-headed microscope by two pathologists (MW, PDR); who were blind to axillary status. In case of disagreement, a final consensus was established.

The validity and reproducibility of most of the following variables are well established¹². However it should be clarified that we did not consider histological tumor type as a workable variable. There are more than thirty tumor types described¹³ and association of patterns would add even more difficulty, hence, to provide homogeneous sets for statistical analysis, we only considered G.

Variable 1: SMC. Briefly, SMC can be defined as an amorphous stromal substance composed of an amphophilic or slightly basophilic vacuolated material found among the collagen fibers (Fig.1). Usually SMC are found intermingled with cells that compose the granulation tissue and within fibrous areas of an early wound healing process. HA is one of its major components as we demonstrated with immunohistochemistry utilizing a HA binding probe¹⁴.

Cases were assessed with a low-power objective, examining both the periphery and center of the lesion. They were recorded as absent or scanty (SMC1), moderate, if at least three foci were found at the periphery (SMC2), and marked (SMC3) when large areas of SMC were observed at the periphery and center of the tumor. In the present work SMC were appraised with hematoxylin-eosin stained slides.

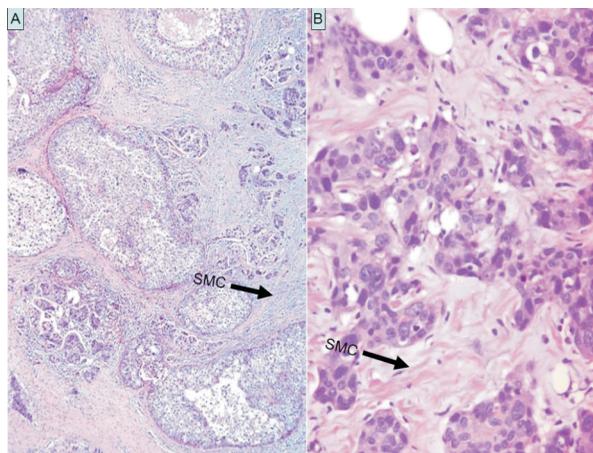


Fig. 1.- 1. A: SMC seen as a slightly basophilic material in the peritumoral stroma. 1. B: SMC found among the collagen fibers.

The level of agreement for this variable was evaluated with kappa statistics in a previous work¹⁴. There was substantial agreement among observers in SMC1 (kappa: 0.77). Greater variations were observed in SMC2 (kappa: 0.53) and SMC3 (kappa = 0.69). Overall kappa showed a substantial level of agreement (kappa: 0.67). There was also a strong correlation between this three grade classification and the staining intensity with the HA binding probe¹⁴.

Variable 2: Fibrotic focus. When there was a well-formed central sclerotic nodule, often associated with acellular hyalinized keloid-like tissue, it was called a fibrotic focus.

Variable 3: TIL. TIL1: Only occasional peritumoral lymphocytes were found, TIL 2: intermediate or dense cellular lymphocytic infiltration.

Variable 4: G. This was evaluated based on the Scarff Bloom Richardson grading system¹⁵. The grade was expressed as G1, G2 or G3.

Variable 5: Embolizations: This refers to peritumoral vascular invasion and was recorded as either positive or negative.

Variable 6: Size. Less than 1cm (T1a-T1b from the TNM Classification of the International Union Against Cancer); 1 to 2cm (T1c) and more than 2cm (T2-T3).

Variable 7: Age. In order to evaluate gradual changes related to aging we subdivided our population into four age groups: 45 years old or younger; 46-55 years; 56-65 years and older than 66.

Variable 8: Multifocality. Present or absent. A tumor was considered multifocal when two or more lesions were located in the same quadrant and distance from each other was less than 5 cm.

Variable 9: Axillary status. The presence of MALN was recorded as positive (including micrometastases) or negative (negative nodes in axillary dissection or negative sentinel lymph node when this technique was performed. We included isolated tumor cells in this group).

The clinicopathological characteristics of the patients are summarized in Table 1.

Starting at our institution in 1999, immunohistochemical determination of hormone receptors and HER2 expression became gradually part of the routine examination, as a result, only 935 and 746 cases had hormone receptors and HER2 determinations performed respectively. Therefore from the 1803 cases, complete analysis was done on 746 patients.

Immunohistochemical studies were performed using antibodies against estrogen receptor (ER) (1D5; 1:35 dilution,

TABLE 1.— *Clinicopathologic characteristics of the 1803 patients*

	Number of cases	%
Axillary status		
Negative	1133	62.6
Positive	670	37.4
SMC		
SMC1	804	44.6
SMC2	561	31.1
SMC3	438	24.3
G		
G1	144	8
G2	1095	60.7
G3	564	31.3
Embolizations		
Absent	1567	86.9
Present	236	13.1
TIL		
TIL1	1349	74.8
TIL2	454	25.2
Age (years)		
<45	316	17.5
46-55	398	22.1
56-65	462	25.7
>66	625	34.7
Missing	2	
Size		
T1a-T1b	384	21.3
T1c	750	41.6
T2-T3	669	37.1
Multifocality		
Absent	1271	70.5
Present	532	29.5
Fibrotic focus		
Absent	1190	66
Present	613	34

DAKO Cytomation), progesterone receptor (PR) (PgR636; 1:40 dilution, DAKO Cytomation) and HER2 (CB11; 1:200 dilution, DAKO Cytomation). All immunostains were performed according to the avidin–biotin method, using tissue sections of 3 µm thickness. Briefly, sections were deparaffinized with xylene and rehydrated in graded ethanol. Antigen retrieval using citrate buffer (pH 6.0) in pressure cooker for ER and PR and in water bath for HER2 was performed. Endogenous peroxidase was blocked by 0.3% hydrogen peroxide for 10 minutes. Sections were incubated with primary antibody for 30 minutes at 37 °C, then with the biotinylated secondary antibody for 20 minutes at 37 °C, and then in avidin–biotin complex for a further 45 minutes. Diaminobenzidine tetrahydrochloride was used as the chromogen and the sections were counterstained with hematoxylin for 1 minute. All incubations were performed at room temperature; between each incubation step, sections were washed with tris-buffered saline buffer. Appropriate positive and negative controls were used throughout.

The staining was evaluated as follows: hormone receptors: +, ++, +++ and ++++ if nuclear staining was observed in

10-50%, 50-70%, 70-90% and more than 90% of tumor cells respectively (+++ and ++++ were merged). No staining or staining less than 10% was considered negative.

For HER2, cases were reevaluated according to the recently published recommendations¹⁶:

Score 0: no staining is observed in invasive tumor cells.

Score 1+: weak, incomplete membrane staining in any proportion of invasive tumor cells, or weak, complete membrane staining in less than 10% of cells.

Score 2+: complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of invasive tumor cells, or intense complete membrane staining in 30% or less of invasive tumor cells.

Score 3+: intense complete membrane staining is observed in more than 30% of invasive tumor cells.

Frequency tables were employed for descriptive analysis. We used the chi-square test to estimate the association of axillary node status with categorical variables and the Mann Whitney *U* test for continuous variables. Statistically significant variables in the univariate studies ($p < 0.05$) were selected for multivariate analysis, which was performed using interactive stepwise logistic regression. Odds ratios (OR) were calculated together with 95% confidence intervals (CI95%).

Statistical analysis was performed by using the Epi Info 6.0 computer program package.

This study was designed and its results presented in accordance with reporting recommendations for tumor marker prognostic studies (REMARK) criteria¹⁷.

Results

Association between the clinicopathological evaluated features and axillary status. Univariate analysis. All variables studied demonstrated statistically significant association with MALN. The figures are depicted in Table 2.

Association between the clinicopathological evaluated features and axillary status. Multivariate analysis. The variables are listed according to their OR (Table 3). Tumors with SMC3 ($p < 0.0001$) and T2-T3 ($p < 0.0001$) were strongly associated with MALN, followed by G3 ($p < 0.0001$), fibrotic focus ($p < 0.0001$), T1c ($p = 0.003$), embolizations ($p < 0.0001$), SMC2 ($p = 0.0002$), TIL2 ($p < 0.0001$), G2 ($p < 0.0001$) and multifocality ($p = 0.002$). SMC3 stood out as the predominant variable related to MALN. Age did not reach statistical significance.

Multivariate analysis taking SMC3 as the dependent variable. It has been reported that an intrinsic relationship exists between lymphatic endothelial cells and functionally related molecules, particularly HA¹⁸, consequently we specifically studied the association between SMC3 and embolizations taking SMC3 as the dependent variable. A strong association with tumor embolizations ($p < 0.0001$) was observed and, as shown above, with MALN ($p < 0.0001$). A negative relationship with age ($p < 0.0001$) was also noted, suggesting a possible hormone influence in the development of SMC.

Evaluation of hormone receptors, HER2 expression and their association with MALN and SMC3. We first studied the association between HER2 and hormone receptors with MALN in a multivariate analysis (Table 4). Both ER

TABLE 2.— Association between the clinicopathological evaluated features and axillary status. Univariate analysis

	Axillary status		p
	Without metastasis (%)	With metastasis (%)	
Size			
T1a-b	345 (89.8)	39 (10.2)	
T1c	514 (68.5)	236 (31.5)	
T2-T3	272 (40.7)	397 (59.3)	<0.0001
SMC			
SMC1	667 (83)	137 (17)	
SMC2	331 (59)	230 (41)	
SMC3	130 (29.7)	308 (70.3)	<0.0001
G			
G1	132 (91.7)	12 (8.3)	
G2	769 (70.2)	326 (29.8)	
G3	226 (40)	338 (60)	<0.0001
Embolizations			
Absent	1070 (68.3)	497 (31.7)	
Present	58 (24.6)	178 (75.4)	<0.0001
TIL			
TIL1	934 (69.2)	415 (30.8)	
TIL2	195 (43)	259 (57)	<0.0001
Fibrotic focus			
Absent	810 (68.1)	380 (31.9)	
Present	321 (52.4)	292 (47.6)	<0.0001
Multifocality			
Absent	872 (68.6)	399 (31.4)	
Present	260 (48.9)	272 (51.1)	<0.0001
Age (years)			
<45	155 (49.1)	161 (50.9)	
46-55	248 (62.3)	150 (37.7)	
56-65	291 (63)	171 (37)	
>66	434 (69.4)	191 (30.6)	<0.0001

+++ (p = 0.02) and PR +++ (p = 0.006) were inversely associated with MALN. In a second step, we investigated the association between SMC3 and hormone receptors. Previous research papers found a positive¹⁰ and inverse association²⁰ of hormone receptors with cytosolic and stromal HA signal respectively. Our analysis, indicated that ER +++ was inversely associated with SMC3 (p < 0.0001) (Table 5).

Discussion

Several pieces of new information emerged from this study: SMC3 were, according to our figures, the most important factor related to MALN; TIL were related to MALN; fibrotic focus was related to MALN; SMC3 were related to tumoral embolizations and inversely associated with ER +++.

TABLE 3.— Association between the clinicopathological evaluated features with MALN. Multivariate analysis. In two cases the age was not available leaving a total of 1801 cases

	OR	p	95% CI
SMC3	6.81	<0.0001	4.7 – 9.8
T2-T3	5.71	<0.0001	3.7 – 8.6
G3	4.9	<0.0001	2.4 – 10.2
Fibrotic focus	3.45	<0.0001	2.5 – 4.7
T1c	2.85	0.003	1.4 – 5.7
Embolizations	2.64	<0.0001	1.7 – 3.9
SMC2	2.59	0.0002	1.9 – 3.4
TIL2	2.47	<0.0001	1.6 – 3.6
G2	2.41	<0.0001	1.7 – 3.2
Multifocality	1.49	0.002	1.1 – 1.9
45-55 years	1	0.98	0.6 – 1.4
55-65 years	0.84	0.4	0.5 – 1.2
>65 years	0.82	0.3	0.5 – 1.1

TABLE 4.— Association between HER2 and hormone receptors with MALN. Multivariate analysis

	OR	p
HER2		
++	1.4	0.11
+++	1.1	0.4
ER		
++	0.7	0.16
+++	0.61	0.02
PR		
++	0.8	0.31
+++	0.54	0.006

TABLE 5.— Association between HER2 and hormone receptors with SMC3. Multivariate analysis

	OR	p
HER2		
++	1.22	0.36
+++	1.14	0.44
ER		
++	0.76	0.31
+++	0.35	<0.0001
PR		
++	1.24	0.36
+++	0.76	0.29

Summarizing, based solely on histological correlations, our findings encompass the entire process of wound healing, which appears to be associated with nodal metastases. This is supported by numerous basic and clinical investigations as it will be exposed in the following discussion.

The results of the first multivariate examination (Table 3) clearly demonstrated the importance of stromal variables in predicting the presence of MALN. SMC as the histological expression of the provisional matrix; fibrotic focus, a scar like lesion in late response to tumor invasion and TIL as the expression of an inflammatory response, were all related to MALN.

We also observed a close association between SMC3 and tumoral embolizations. Since HA is one of the major constituents of SMC¹⁴, our findings allow us to infer that this molecule is involved in the promotion of embolizations. Experimental confirmation of this phenomenon was stated in two recent reports^{21, 22}.

Hence, if SMC3 mirror these aggressive lesions, an epithelial - SMC interaction might be responsible for such increased prometastatic activity. It appears that SMC are the histological expression of a major pathogenetic mechanism, providing a fertile soil for the cascade of changes that lead to metastases²³. Yet, we do not have a clear idea of the true nature of the SMC and cannot unambiguously determine cause and effect. It is possible that they represent manifestations of another underlying process such as increased stromal degradation secondary to proteolytic enzymes. It should be stressed that SMC inevitably induce structural abnormalities in the tumor microvasculature, leading to a reduced oxygen transport capacity and hypoxia as occurs in early wound healing²⁴. In turn, hypoxia can stimulate the production of HA and the activity of hyaluronidase, responsible for degrading HA, promoting angiogenesis as a compensation mechanism²⁵. Moreover, it was recently reported with a tissue microarray technique, a positive correlation between intense stromal HA signal and high nitric oxide synthases expression in human breast cancer. This observation points to high concentrations of reactive oxygen species at the SMC, which also react with HA resulting in chain cleavage²⁰. Therefore, in all likelihood, SMC are composed, besides putative growth promoting factors, of a mixture of high molecular weight hyaluronan (HMW-HA) and low molecular weight hyaluronan (LMW-HA) chains induced by HA degradation.

In our previous work, a correlation (not statistically significant) was observed between mortality and SMC2-3¹⁴. However, Corte et al found decreased cytosolic HA in poorly differentiated tumors and high HA levels associated with significantly longer relapse-free survival¹⁹. Their measurements were done predominantly with an immunological radiometric assay selecting a HMW-HA population. In their data HA levels were significantly higher

in well differentiated diploid ER positive tumors, while in our series SMC3 correlated with G3 and inversely with ER expression (Table 4). It seems that both methodologies select different micro-environments with also different cellular populations. What lies in our view behind these opposite findings, are evidences of a dual role of HA. HMW-HA contents may mean less HA turnover, with lesser tissue destruction and remodeling. Tissue destruction is the main cause of HA degradation²⁵. Very likely the population selected by Corte are tumors with a reduction of HA degradation while SMC are the histological expression of such occurrence. There are several reports documenting a relationship between HA degradation and tumor invasion^{26, 27}. Alternatively the HMW-HA maintains homeostasis and potentially down-regulates inflammation. The early pioneering work of Herrera-Gayol and Jothy demonstrated that intratumoral injection of HMW-HA does not induce stimulation but tumor regression in xenotransplanted breast cancer²⁸, an observation supported by numerous literature data that prove that the effect of HA is closely dependent on its molecular mass.

This dichotomous behavior of HA has already been reported²⁹. However this "Jekyll and Hide" effect has not been clearly referred to neoplastic disease yet.

Harmonizing to these events is the observation that TIL, other actors of the stromal remodeling process behave as prometastatic (Table 3). The histological observations consistently demonstrate TIL interspersed within the spaces opened by SMC. It is known that LMW-HA enhances inflammation³⁰. However, lymphocytes may become compromised once within the SMC^{31, 32} or may adversely adapt to the suppressive environment to promote growth and metastases¹⁰. This is probably due to the release of pro-inflammatory mediators. Chavey et al³³ found in a population of 105 cases that elevated expression of chemokines in breast lesions correlated with marked leukocyte infiltration. They inferred that chemokines could be involved in tumor aggressiveness because ER negative cases exhibit high cytokine content. However they did not find a correlation between chemokine expression and MALN. Our data insinuate that such correlation does exist.

Lastly, our results show a strong relationship between fibrotic focus and MALN (Table 3). Reinforcing this, an association between fibrotic focus and intratumoral hypoxia was detected immunohistochemically³⁴. We believe that fibrotic focus is itself an expression of the wasting product of the stromal process with the active course of action being carried on within the SMC.

All these pieces of information converge to two crucial works. The first is Dvorak's hypothesis³⁵. He propose a never ending process of stromal remodeling, pointing that tumors are like "wounds that never heal." The second is the work of Chang et al³⁶. They document that a gene expression program related to wounding is frequently activated in invasive carcinomas and confers increased risk of metastases.

Undoubtedly, behind these stromal changes there is an intricate balance between HA synthesis and degradation with a desmoplastic reaction following the active chronic inflammation. Even though a cause and effect relationship remains to be verified, these findings provide not only new pathological indicators of aggressiveness but a possible new, previously unappreciated, therapeutic target. HMW-HA, although associated with less aggressive carcinomas, appears to form a protective cellular coat. Cross-linked HA matrix was found to create a microenvironment favoring cells resistance to antimetabolic agents³⁷. HMW-HA induces antiapoptotic as well as drug resistance signals^{38, 39}. Therefore, targeting HA, in order to dissolve the protective pericellular coat, might render malignant cells much more drug sensitive, more responsive to the effect of the stromal dendritic cells activated by LMW-HA fragments⁴⁰ and to the oxidative stress of reactive oxygen species as well²⁰. It is attractive to suggest that hyaluronidase targeted against SMC, may liquefy this protective pericellular coat cutting the vicious circle of the ongoing tissue destruction. Experimental and clinical evidence demonstrated an effect of hyaluronidase as a single agent⁴¹. It was also determined synergistic activity of hyaluronidase with other chemotherapeutic agents⁴²⁻⁴⁴.

In summary, this study underlines the importance of the stromal factor at the time of tumor evaluation. It demonstrates that the stroma suffers histological modifications that reflect a process, analogous to wound healing, where chemokines producing inflammatory cells, desmoplasia, hypoxia, oxygen reactive species and hyaluronan appear to confabulate in order to promote tumor spread and metastases. These changes, when carefully assessed, can predict the metastatic potential of a particular neoplasm and potentially avoid unnecessary surgical procedures.

Conflicts of interest: The authors declare that they have no conflict of interest.

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47. "The surgeon is often intolerant and the internist self sufficient."

47. "El cirujano es a menudo intolerante y el internista autosuficiente."

William J. Mayo (1861-1939)

Aphorisms of Dr. Charles Horace Mayo (1865-1939) and Dr. William James Mayo. Collected by Fredrick A. Willis. Rochester (Minnesota): Mayo Foundation, 1990. p 56