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Immunopathological Role of FAS-FASL Apoptotic Pathway in *H.pylori* CagA ^{Positive} Associated Chronic Atrophic Gastritis in Iraqi Patients

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Abstract
Chronic atrophic gastritis (CAG) characterized by chronic inflammation of gastric mucosa with loss of gastric glandular cells and replacement by intestinal-type epithelium and fibrous tissue. CAG may be associated with *Helicobacter pylori* infection. The main Objective was to determine the role of FAS-FASL apoptotic pathway in *H.pylori* CagA positive associated chronic atrophic Gastritis .Forty five CAG patients and (20) control were chosen. Multiple gastric mucosal biopsies taken for rapid urease test and slides preparation from paraffin blocks. Biotinylated DNA probe for human FAS, FASL, genes used for detection of their expression in tissue lymphocytes and gastric epithelial cells using ISH technique. Biotinylated DNA probe for H.pylori Cag A gene used for detection of its expression in gastric epithelial cells. Insitu CagA was detected in (46.66%) of CAG cases. The toxin was not detected among control .Tissue FAS mRNA expression was higher than lymphocytes FAS mRNA among CagA positive and CagA negative CAG cases. Tissue infiltrating lymphocytes (TILs) FAS expression was higher among Cag Apositive than Cag A negative CAG cases. Significant difference in FASL mRNA between Gastroduodenal tissue (GDT) and TILs among Cag A positive and Cag A negative CAG cases was detected. Lymphocyte grade has significant positive correlation with tissue FAS mRNA expression (p=0.005).Tissue FAS mRNA expression has significant positive correlation with CagA (p=0.000). Lymphocyte FAS mRNA expression has significant positive correlation with lymphocyte FASL mRNA expression (p=0.03) and CagA (p=0.000) .Tissue FasL has significant negative correlation with CagA (p=0. 001). Lymphocyte FASL mRNA expression has significant positive correlation with CagA (p=0.008).In Conclusions, FAS-FASL pathway play vital role in Immunopathology of *H.pylori* Cag A+ associated gastritis that leads finally to atrophic changes.
Keywords: atrophic gastritis, Fas, FasL, H.pylori

Introduction:

Chronic Atrophic gastritis (CAG) is a histopathological entity characterized by chronic inflammation of the gastric mucosa with loss of gastric glandular cells and replacement by intestinal-type epithelium and fibrous tissue. CAG may be associated with Helicobacter pylori infection as well as unidentified environmental factors and autoimmunity directed against gastric glandular cells (1) .Apoptosis, programmed cell death, is essential in development and homeostasis of multicellular organisms. In addition, it may also serve as a defense mechanism against bacterial and viral infections (2). Apoptosis can be mediated through activation of the CD95 receptor (CD95) (APO-1/Fas) and ligand system (3). There is an evidences of possible relationship among the development of atrophic gastritis and the apoptotic process that takes place in the epithelial cells of stomach and duodenum (4).during the disease process there is an imbalance between epithelial cell proliferation and death from apoptosis ,thus disregulating the normal cell cycle and initially lead to gastritis ,and this may become gastric atrophy ,and then subsequently metaplasia,dysplasia and even cancer (4,5). Helicobacter pylori (H.pylori) is considered as an important etiologic factor in the development of chronic gastritis and ulcer disease (6,7) . In toxigenic H. pylori–induced chronic gastritis, surface epithelial damage, erosions, and atrophy of the gastric mucosa as well as increased epithelial proliferation are striking histological features. Epithelial proliferation does not seem to be counterbalanced by epithelial necrosis in H. pylori– induced gastritis suggesting that apoptosis may account for the apparent cell loss in chronic gastritis (8) .In biopsies taken from patients with H. pylori–associated chronic atrophic gastritis ,duodenal ulcer, apoptosis of gastric epithelium was increased and involved upregulation of CD95 expression in surface epithelium, lamina propria lymphocytes, and pyloric gland cells and increase in CD95L expression in lymphocytes and gastric epithelial cells (4, 9). The main Objective was to determine the role of FAS-FASL Apoptotic pathway in H.pylori CagA positive associated chronic atrophic gastritis.

Materials and methods

2.1. Patients:

The subjects of the present study selected from Patients attending the gastroenterology unit of digestive and hepatic diseases teaching hospital in Baghdad. Ethics committee of Diyala University, college of Medicine, approved the present study. At first the aim of study was explained for all participants and after obtaining

their oral consent they have been studied .The subjects were diagnosed as having atrophic gastritis by upper gastrointestinal endoscopy from May 2009 to December 2010.Forty five patients suffering from chronic atrophic gastritis were chosen. Twenty apparently healthy volunteers enrolled as control.

2.2. Exclusion criteria used in the study

Patients who had undergone or were currently undergoing *H.pylori* eradication therapy, Patients who had received antiulcer treatment in the last three months , and still receiving proton-pump inhibitors (PPI) or H 2 receptor blockers. Patients who had received nonsteroidal anti-inflammatory drugs (NSAIDs) within two months before the examination. The result of rapid urease test was negative; all were excluded from investigation **(10).**

2.3. Sample collection:

Patients were fasted for at least eight hours before endoscopic examination. Endoscopic examination performed under local pharyngeal anesthesia. Using fiber optic endoscope, multiple mucosal biopsy specimens taken via sterile standard biopsy forceps from the stomach (inflamed area of gastric mucosa). One-biopsy specimens used for rapid urease test (RUT) for detection of *H.pylori* in tissue sample. Other biopsy specimen used for gram stain. Rest biopsy specimens fixed with 10% buffered formalin for preparation of paraffin embedded tissue blocks to prepare slides for histological examination using haematoxylin-eosin. Biopsy specimens obtained from the antrum used to evaluate the grades of lymphocytes infiltration. The presence of *H.pylori* in tissue section was established by typical appearance on scanning along the mucosal surface and the individual gastric pits **(10)**.

2.4.DNA Probes and DNA Probe hybridization/Detection System :

a)Biotinylated long DNA probe for human Fas Gene, Cat. No.: IH-60047(fas-6001-B). (Maxim biotech-USA);b) Biotinylated long DNA probe for human FasL gene, Cat. No. IH-60031) (Maxim biotech-USA).*c)* Biotinylated long DNA probe for H.pylori/ Cag A Gene, Cat. No.: IH-60061(HPY-6001-B) (Maxim biotech-USA).d) The DNA Probe hybridization/Detection System – In Situ Kit (Maxim biotech-USA).

2.5. Biopsy urease test (BUT):

The biopsies were placed directly into the rapid urea medium at the time of endoscopy**.** Positive results indicated by development of a pink-red or red-violet color **(11)**.

2.6. Insitu hybridization procedure

Human (Fas and FasL) gene as well as Cag A gene expression for H.pylori were detected in 4-6 µm thickness serial gastric mucosal tissue sections fixed on positively charged slides .According to Maxim biotech instruction manual (12). The examination and scoring were done under light microscope by a pathologist at power X400 according to the scoring system used by (10) in which negative results considered if less than 5% of examined cells were positive. low positive score was considered if 5-25% of examined cells were positive. Intermediate positive score considered if 26-50% of examined cells were positive. High positive score considered if >50% of examined cells were positive.

2.7. Scoring system for lymphocytes [(13)]

Lymphocyte infiltration: Grading scale from 0 to 3, based on both lymphocyte and plasma cell infiltration. Grade 0 considered if normal cellular finding detected. Grade 1 considered if in case of low inflammation, Grade 2 for Moderate inflammation and Grade 3 indicate heavy inflammation.

3. Statistical analysis:

Data analysis performed using the following tests:

1. T-test used to find out the significance of differences between two groups that composed from continuous variables.

2. Pearson test for correlation used for non-categorical data. The level of significance was 0.05(two-tail) in all statistical testing; significant of the level of confidence limits was 0.095.Statistical analysis performed using SPSS for windows TM version 14.0, and Microsoft EXCEL for windows 2007.

Results:

Forty five patients diagnosed endoscopically and histopathologically have CAG with the mean age (39.26±17.03) years were enrolled in the present study. CAG detected in (73.3%) of males versus (26.7%) among female as shown in table (1) . As shown in table (2) and figure (1 -A), *Insitu* CagA Cytotoxin detected in 21(46.66%) out of 45.The toxin was not detected among control cases. *In situ* FAS mRNA expression level evaluated by using certain scoring system. Positive expression of FAS mRNA defined as expression in 5% or more of GDT. Positive expression of tissue FAS mRNA was detected in 21/45 case of $CagA^{positive}$ CAG (46.66%).Tissue expression of Fas mRNA was more than 50% in 9/45 (20%) of $CagA^{positive}$ CAG cases. Among $CagA^{negative}$ tissue expression of Fas mRNA was more than 50% in 12/45 (26.66%). Intermediate expression of tissue Fas mRNA was identical in both $CagA^{positive+}$ and $CagA^{negative}$ CAG cases in which 12(26.66%) out of 45 case gave same score of expression. Expression of lymphocyte Fas mRNA was more than 50% in 6/45 (13.33%) of $CagA^{positive}$ CAG cases. Intermediate expression of lymphocyte Fas mRNA was detected in 12 /45 (26.66%) of $CagA^{positive}$ CAG cases compared with 9/45 (20%) of $CagA^{negative}$ cases. Up to (70%)of normal control cases tissue Fas mRNA

expression was negative and only in (30%) of cases low level of expression was detected on the other hand no detectable level of Fas mRNA expression was found regarding to lymphocytes. Details of tissue Fas mRNA expression and scoring system was explained in table (3) and figure(1-C).Based on T-test, there is a significant differences between GDT and TILs ($p<0.05$) in FAS mRNA expression among cag A ^{positive} and cag A ^{negative} CAG cases . There is a significant difference between Cag $^{\mathbf{positive}}$ and Cag A $^{\mathbf{negative}}$ Cases regarding over expression of tissue FAS mRNA ($p<0.05$) as shown in table (4)

In the present study we investigate *in situ* FASL mRNA expression in 45 cases of CAG. *In situ* FASL mRNA expression level evaluated by using certain scoring system .Figure (1-D) shows the typical *in situ* staining for tissue FASL mRNA. Less than 5%of stained cells considered negative for CAG cases. All other percentage of expression considered positive .positive expression of FASL mRNA defined as expression in 5% or more of GDT. Positive expression of tissue FASL mRNA was detected in 21/45case of *Cag A* $^{\mathbf{positive}}$ CAG (46.66%).Tissue expression of FasL mRNA was more than 50% in 3/45 (6.66%) of *Cag A* $^{\mathbf{negative}}$ CAG cases compared with *Cag A* $^{\mathbf{positive}}$ cases in which high level of expression was not detected. details of expression were explained in were explained in table (5).Based on T-test , significant differences in FASL mRNA expression between GDT and TILs among *Cag A* $^{\mathbf{positive}}$ as well as Cag A ^{negative} cases .There was no significant difference between *Cag A* $^{\mathbf{positive}}$ and Cag A $^{\mathbf{negative}}$ Cases regarding tissue FASL mRNA over expression ($p>0.05$) as shown in table(6). Comparative evaluation of FasL mRNA expression in GDU,CAG and control group we found that there is a significant differences between control group and *H.pylori Cag A*^{positive} CAG regarding tissue expression of FasL mRNA ($p <0.05$) .Significant differences between control group and *H.pylori* CagA^{negative} CAG regarding tissue expression of FasL mRNA ($p <0.05$) as shown in table (6).

As shown in tables (7) Lymphocyte grade has significant strong positive correlation with tissue FAS mRNA expression (r= 0. 409, p=0.005) .tissue FAS mRNA expression has significant strong positive correlation with CagA (r= 0.540, p=0.000) . Lymphocyte FAS mRNA expression has significant strong positive correlation with lymphocyte FASL mRNA expression (r= 0. 310, p=0.03) and CagA (r= 0.511, p=0.000) .Tissue FasL has significant strong negative correlation with CagA(r= -0.484, p=0. 001). Lymphocyte FASL mRNA expression has significant strong positive correlation with CagA (r= 0.390,p=0.008).

Table (1): patients' and Control Group Demography

Subjects demography		Clinical presentation	
		CAG	Control
Age	Minimum	18	23
	Maximum	75	58
	Mean± SD	39.26 ± 17.03	38 ± 10.89
Gender	Male	33 (73.3%)	10(50%)
	Female	12(26.7%)	10(50%)
Total No.		45(100%)	20(100%)

SD: Standard deviation

Table (2): Frequency distribution of Insitu CagA Cytotoxin among CAG cases versus control

Clinical presentation	Insitu Cag A detection		
	Positive	Negative	Total
CAG	21(46.66%)	24 (53.33%)	45
Control	0(0%)	10(100%)	10

Table (3): Frequency distribution of tissue versus lymphocytes insitu expression of FAS mRNA in Cag A $^{\mathbf{positive}}$/Cag A negative CAG Patients

CagA In situ	Marker CD95(APO1/FAS)	Negative Grade	Positive Grade			Total No.
			Low	Intermediate	High	
		< 5%	5-25%	26-50%	>50%	
Cag positive	Tissue FAS	0(0%)	0(0%)	9(20%)	12(26.66%)	21(46.66%)
	Lymphocyte FAS	0(0%)	3(6.66%)	12(26.66%)	6(13.33%)	
Cag negative	Tissue FAS	0(0%)	3(6.66%)	15(33.34%)	6(13.33%)	24(53.33%)
	Lymphocyte FAS	0(0%)	15(33.34%)	9(20%)	0(0%)	
Control	Tissue FAS	14(70%)	6(30%)	0(0%)	0(0%)	20 (100%)
	Lymphocyte FAS	0(0%)	0(0%)	0(0%)	0(0%)	
Total no of CAG cases		45(100%)				

Table (4): Tissue versus lymphocytes insitu expression of FAS mRNA in Cag Apositive/Cag Anegative CAG Patients.

Marker CD95(APO1/FAS)	Cag A Positive	Cag A Negative	Total No.	T-test P value
Tissue FAS mean ± SE*	21(46.66%) 66±3.45	24(53.33%) 45.62±3.45	45	P<0.05
Lymphocyte FAS mean ± SE*	21(46.66%) 38.57±6.28	24(53.33%) 22±6.2	45	P<0.05
T-test (p- value)	P<0.05	P<0.05		

Table (5): Frequency and distribution of tissue versus lymphocytes insitu expression of FASL mRNA according to scoring system in Cag Apositive/ Cag A negative CAG Patients.

CagA In situ hybridization	Marker CD95L(FASL)	Negative	Positive			Total No.
		Grade	Grade			
			Low	Intermediate	High	
		< 5%	5-25%	26-50%	>50%	
Cag A^{positive}	Tissue FASL	0(0%)	15(33.33%)	6(13.33%)	0(0%)	21(46.66%)
	Lymphocyte FASL	0(0%)	0(0%)	6(13.33%)	15(33.33%)	
Cag A ^{negative}	Tissue FASL	3(6.66%)	9(20%)	9(20%)	3(6.66%)	24(53.33%)
	Lymphocyte FASL	0(0%)	3(6.66%)	6(13.33%)	15(33.33%)	
control	Tissue FASL	14(70%)	6(30%)	0(0%)	0(0%)	20(100%)
	Lymphocyte FASL	0(0%)	0(0%)	0(0%)	0(0%)	
Total no of CAG cases		45(100%)				

Table (6): Tissue versus lymphocytes insitu expression of FASL mRNA in Cag A positive/Cag Anegative CAG Patients and control group.

Marker CD95L(FAS L)	Cag Apositive	Cag A negative	T-Test p value	Control group	T-Test p value
Tissue FASL mean ± SE*	21(46.66%) 19.71±4.88	24(53.33%) 25±6.79	p>0.05	3.3±0.66	p>0.05
Lymphocyte FASL mean ± SE*	21(46.66%) 78.57±8.51	24(53.33%) 51±6.47	P<0.05	ND*	ND*
p- value	P<0.05	P<0.05		ND*	ND*

Table (7) : Correlations among tissue and cellular markers of CAG cases:

Parameter		Tissue Fas	lymphocyte FAS	Tissue FASL	lymphocyte FASL	CagA
Lymphocyte grade	r	0.409**	-0.131	-0.283	-0.125	0.094
	P-value	0.005	0.391	0.059	0.412	0.537
Tissue Fas	r		0.116	-0.203	0.095	0.540**
	P-value		0.447	0.180	0.534	0.000
Lymphocyte Fas	r			-0.033	0.310*	0.511**
	P-value			0.831	0.038	0.000
Tissue FasL	r				0.075	-0.484**
	P-value				0.622	0.001
Lymphocyte FasL	r					0.390**
	P-value					0.008

r = Pearson Correlation

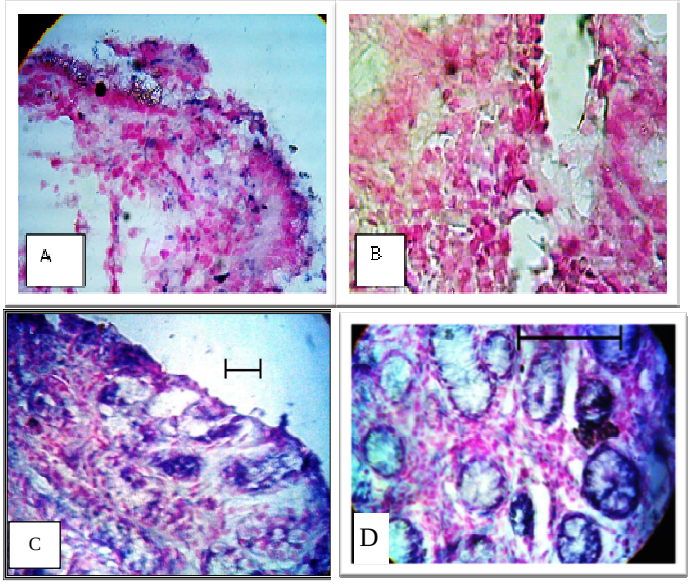

Figure (1):
A) *In situ* hybridization for CagA Positive *H.pylori in* gastric tissue section .staining by BCIP/NBT (bluish purple) counterstained with nuclear fast red. Bar size=50µm.
B) negative expression .Bar size=50µm.
C) *In situ* hybridization for human Fas in gastric atrophy tissue section mainly in gastric glands and inflammatory cell infiltrates staining by BCIP/NBT (bluish purple) counterstained with nuclear fast red. Bar size=50µm.
D) *In situ* hybridization for human FasL in gastric atrophy tissue section .staining by BCIP/NBT (bluish purple) counterstained with nuclear fast red. Bar size=50µm.

Discussion:

In the present study the prevalence of CAG among males and females was (73.3%) and (26.7%) respectively .This come in concordance with that reported among Canadian patients (44.1%) for males and (55.9%) for females (14) and Italian patients males represent (43.22%) and (56. 78%) were females(15). Others (16) were record high incidence of CAG among females (63%) compared with (37%) in males.

the possible reason for such difference may be related to study design which was an epidemiological study and focus on the prevalence of H.pylori infection in relation with gender and the size of community under investigation that give a chance for difference in sample size of each gender type which is apparently larger in case of females in European communities .Emotional stress factor may have a role in susceptibility to H.pylori infection .Gender differences to H.pylori induced gastroduodenal diseases and in disease susceptibility may reflect differences in immune responses, hormonal effects and sex linked genetic factors and differences in bacterial colonization (17) .The ability of H.pylori to induce CAG in the present study was related to several H.pylori related virulence factors that contribute in dissimilar ways in gastric mucosal damage. Among these factors are urease and flagella that known to be required for the colonization and survival of *H. pylori* in the human stomach. *H. pylori* manufacture large amounts of urease that helps to break down urea into carbon dioxide and ammonium, which neutralizes the acid during colonization of the stomach. Urease plays a dual role by making protection of H.pylori from gastric acid and induction of apoptosis in gastric cells as well as inhibits gastric somatostatin release in animals, which could alters the digestion physiology (18; 19).

The present study determine a significant correlation between CAG induced by H.pylori CagApositive and the severity of lymphocytes infiltration in gastric mucosa and subsequently with the severity of atrophic changes compared with CAG induced by H.pylori Cag Anegative ,that cause less extensive inflammatory changes in gastric mucosa and this finding come in accordance with others (20 ; 21; 22) . The results of the present study give a primary indication about *H.pylori* vital role as we notice that there was obvious effect on the grade of FAS and FASL expression in gastric tissue and i lymphocytes infiltration among cases that harbored this pathogen in gastroduodenal biopsies compared with normal apparently healthy control. This could be related to several factors belongs to molecular interaction between pathogen and mucosal surface of its host. The production of CagA in the present study give an indication that *H.pylori* poses an active Cag pathogenicity island encoding genes that may activate or even inhibit adherent factors which are utilized by H.pylori for its adhesion to the gastric epithelial cells possibly during the processes of adhesin synthesis, transportation, secretion and activation this collectively play a central role in the final scenario of interaction between gastric mucosa and H.pylori. Whenever pathogen have potent Cag PAI, bacterial adhesion, and good signals will be received by the gastric epithelial cells which reflect its response by increasing MHCII expression to play as antigen presenting cell and strong Th1 response will be occur with obvious IFNγ secretion which act as good stimulator for up-regulation of FAS and even FASL in gastroduodenal tissue (GDT) and tissue infiltrating lymphocytes (TILs)(23).

In the present study in CAG ,CagA positive *H. pylori* –infected mucosa ,the expression of CD95 and CD95L was upregulated in surface epithelium, lamina propria lymphocytes with significant differences recorded

between gastric tissue and infiltrating lymphocytes as well as between CagApositive and CagAnegative infected gastric tissue and this come in accordance with studies around the world (24,25,26,27), increased levels of CD95L mRNA found by insitu in lamina propria cells suggest that lymphocytes in the lamina propria express CD95L and induce apoptosis via CD95L in CD95-positive neighboring cells.

Expression of Fas and FasL in gastric tissue and infiltrating lymphocytes support our opinion that apoptosis of gastric epithelial cell not only induced by CD95L-expressing lymphocytes but also by CD95L-expressing epithelial cells. CD95L expressed on gastric epithelial cells in a membrane-bound form mediate apoptosis by "fratricide" interacting with CD95 on adjacent epithelial cells as well as through suicide of the CD95L-CD95–expressing cell itself . Upregulation of Fas-FasL in gastric tissue may be related to the cytokine microenvironment or H.pylori factors other than Cag and Vac .This opinion was come in agreement with (28,29,30) they stated that CagA producing *H. pylori* strains are associated with increased Th1 cytokines production in the gastric mucosa and in gastric epithelial cell lines, Among these inflammatory cytokines, IL-1, IL-2, TNF-α, and IFNγ that have been shown to up-regulate the expression of Fas Ag in cell lines and in gastroduodenal biopsies. IFNγ and TNF- α, and *H. pylori* infection activate transcription factors in gastric epithelial cells that bind to the NF-kB and AP-1 regulatory sites (30,31) Since these regulatory sites are located in the 59 region of the Fas gene, it is possible that both can contribute to the induction of Fas expression that were studied using ISH detection technique (32). However, our results showed that *H. pylori* strain, which lacks the cag PAI, induced Fas expression, although to a lesser degree. Th1 cytokines can not only injure the gastric epithelium directly (33,34) but also accelerate epithelial damage by modulating Fas/FasL interactions (35;36).

The frequency of epithelial apoptosis found to be significantly lower in noninflamed gastric mucosa. Mannick, et al., (1996)(37) agree with result of the present study, they describe increased apoptosis of epithelial cells in the neck region of the gastric glands that decreased after eradication therapy in H. pylori-infected subjects. The presence of apoptotic epithelial cells suggests that the epithelial barrier may be compromised. This could contribute to increased permeability and a breakdown in the cytoprotective mechanisms that guard the epithelium against damage to luminal acid and pepsin. Studies of (38,39) were giving bright evidence about the role of H.pylori infection in apoptosis induction of gastric epithelial cells. They found a significant increase in apoptotic index only in H.pylori infected subjects, in comparison to uninfected subjects. However, these studies did not examine the effects of *H. pylori* CagA positive and CagAnegative strains separately. In brief the results of (34,38, 39) which represent a results from different area around the world come in agreement with the results of the present study give a supportive evidence about the main role of Cag A positive H.pylori in apoptosis induction of gastric epithelial cells. The possible reason for differences between the results of the present study and other conflicting studies might be related to study design. Our study strategy depends on the comparative evaluation of the possible effect of CagApositive H.pylori on CAG cases compared with normal apparently healthy volunteers, other conflicted clinical studies like study of (40) who depends on the single clinical presentation type, which is chronic gastritis, beside differences in population under focus, ethnic groups, and geographical area of the study.

In Conclusions, FAS-FASL pathway play vital role in Immunopathology of H.pylori Cag A+ associated gastritis that leads finally to atrophic changes.

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