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## Common genetic variants in the CLDN2 and PRSS1-PRSS2 loci alter risk for alcohol-related and sporadic pancreatitis

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## Common genetic variants in the *CLDN2* and *PRSS1-PRSS2* loci alter risk for alcohol-related and sporadic pancreatitis

David C. Whitcomb<sup>1,2,3,128</sup>, Jessica LaRusch<sup>1</sup>, Alyssa M. Krasinskas<sup>4</sup>, Lambertus Klei<sup>5</sup>, Jill P. Smith<sup>6</sup>, Randall E. Brand<sup>1</sup>, John P. Neoptolemos<sup>7</sup>, Markus M. Lerch<sup>8</sup>, Matt Tector<sup>9</sup>, Bimaljit S. Sandhu<sup>10</sup>, Nalini M. Guda<sup>9</sup>, Lidiya Orlichenko<sup>1</sup>, Alzheimer's Disease Genetics Consortium<sup>11</sup>, Samer Alkaade<sup>12</sup>, Stephen T. Amann<sup>13</sup>, Michelle A. Anderson<sup>14</sup>, John Baillie<sup>15</sup>, Peter A. Banks<sup>16</sup>, Darwin Conwell<sup>16</sup>, Gregory A. Coté<sup>17</sup>, Peter B. Cotton<sup>18</sup>, James DiSario<sup>19</sup>, Lindsay A. Farrer<sup>11,20</sup>, Chris E. Forsmark<sup>21</sup>, Marianne Johnstone<sup>7</sup>, Timothy B. Gardner<sup>22</sup>, Andres Gelrud<sup>1</sup>, William Greenhalf<sup>7</sup>, Jonathan L. Haines<sup>11,23</sup>, Douglas J. Hartman<sup>4</sup>, Robert A. Hawes<sup>18</sup>, Christopher Lawrence<sup>18</sup>, Michele Lewis<sup>24</sup>, Julia Mayerle<sup>8</sup>, Richard Mayeux<sup>11,25</sup>, Nadine M. Melhem<sup>5</sup>, Mary E. Money<sup>26</sup>, Thiruvengadam Muniraj<sup>27</sup>, Georgios I. Papachristou<sup>1</sup>, Margaret A. Pericak-Vance<sup>11,28</sup>, Joseph Romagnuolo<sup>18</sup>, Gerard D. Schellenberg<sup>11,29</sup>, Stuart Sherman<sup>17</sup>, Peter Simon<sup>8</sup>, Vijay K. Singh<sup>1</sup>, Adam Slivka<sup>1</sup>, Donna Stolz<sup>2</sup>, Robert Sutton<sup>7</sup>, Frank Ulrich Weiss<sup>8</sup>, C. Mel Wilcox<sup>30</sup>, Narcis Octavian Zarnescu<sup>1</sup>, Stephen R. Wisniewski<sup>31</sup>, Michael R. O'Connell<sup>1</sup>, Michelle L. Kienholz<sup>1</sup>, Kathryn Roeder<sup>32</sup>, M. Michael Barmada<sup>3</sup>, Dhiraj Yadav<sup>1</sup>, Bernie Devlin<sup>3,5,128</sup>, Marilyn S. Albert<sup>33</sup>, Roger L. Albin<sup>34,35</sup>, Liana G. Apostolova<sup>36</sup>, Steven E. Arnold<sup>37</sup>, Clinton T. Baldwin<sup>38</sup>, Robert Barber<sup>39</sup>, Lisa L. Barnes<sup>40,41</sup>, Thomas G. Beach<sup>42</sup>, Gary W. Beecham<sup>28</sup>, Duane Beekly<sup>43</sup>, David A. Bennett<sup>40,44</sup>, Eileen H. Bigio<sup>45</sup>, Thomas D. Bird<sup>46</sup>, Deborah Blacker<sup>47,48</sup>, Adam Boxer<sup>49</sup>, James R. Burke<sup>15</sup>, Joseph D. Buxbaum<sup>50,51,52</sup>, Nigel J. Cairns<sup>53</sup>, Laura B. Cantwell<sup>29</sup>, Chuanhai Cao<sup>54</sup>, Regina M. Carney<sup>55</sup>, Steven L. Carroll<sup>56</sup>, Helena C. Chui<sup>57</sup>, David G. Clark<sup>58</sup>, David H. Cribbs<sup>59</sup>, Elizabeth A. Crocco<sup>60</sup>, Carlos Cruchaga<sup>61</sup>, Charles DeCarli<sup>62</sup>, F. Yesim Demirci<sup>3</sup>, Malcolm Dick<sup>63</sup>, Dennis W. Dickson<sup>64</sup>, Ranjan Duara<sup>65</sup>, Nilufer Ertekin-Taner<sup>64,66</sup>, Kelley M. Faber<sup>67</sup>, Kenneth B. Fallon<sup>56</sup>, Martin R. Farlow<sup>68</sup>, Steven Ferris<sup>69</sup>, Tatiana M. Foroud<sup>67</sup>, Matthew P. Frosch<sup>70</sup>, Douglas R. Galasko<sup>71</sup>, Mary Ganguli<sup>5</sup>, Marla Gearing<sup>72,73</sup>, Daniel H. Geschwind<sup>74</sup>, Bernardino Ghetti<sup>75</sup>, John R. Gilbert<sup>28</sup>, Sid Gilman<sup>34</sup>, Jonathan D. Glass<sup>76</sup>, Alison M. Goate<sup>61</sup>, Neill R. Graff-Radford<sup>64,66</sup>, Robert C. Green<sup>77</sup>, John H. Growdon<sup>78</sup>, Hakon Hakonarson<sup>79</sup>, Kara L. Hamilton-Nelson<sup>28</sup>, Ronald L. Hamilton<sup>80</sup>, Lindy E. Harrell<sup>58</sup>, Elizabeth Head<sup>81</sup>, Lawrence S. Honig<sup>82</sup>, Christine

<sup>128</sup>Correspondence should be addressed to D.C.W. (whitcomb@pitt.edu) or B.D. (devlinbj@upmc.edu)..

### AUTHOR CONTRIBUTIONS

Writing Group: A.M.K., B.D., D.C.W., J.L., L.K., M.L.K.

Project design, management, and coordination: B.D., D.C.W., D.Y., J.P.N., M.M.B., M.M.L., N.M.M., S.R.W.

Sample collection and phenotyping: A.B., A.C.M., A.C.N., A.G., A.G.S., A.I.L., A.J.S., A.K., A.M.G., A.P., A.P.L., A.R., A.S., B.G., B.L.M., B.N.V., B.R., B.S.S., B.T.H., C.A.M., C.B.W., C.Cao, C.Cruchaga, C.D., C.E.F., C.E.Y., C.F.L., C.G.L., C.L., C.M.H., C.M.W., C.R., C.T.B., D.A.B., D.Beekly, D.Blacker, D.C., D.C.Marson, D.C.Mash, D.C.W., D.G.C., D.H.C., D.H.G., D.R.G., D.W.D., D.W.T., D.Y., E.A.C., E.D.R., E.H., E.H.B., E.H.K., E.M., E.M.R., E.P., E.R.M., F.M., F.M.L., F.U.W., F.Y.D., G.A.C., G.A.J., G.I.P., G.J., G.L., G.W.B., H.C.C., H.H., H.J.R., H.P., H.V.V., J.A.K., J.A.Schneider, J.A.Sonnen, J.B., J.B.L., J.C.M., J.C.T., J.D., J.D.B., J.D.G., J.E.P., J.F.Q., J.H.G., J.H.K., J.L.L., J.M., J.M.O., J.M.R., J.P.N., J.P.S., J.P.V., J.Q.T., J.R., J.R.B., J.R.G., J.R.M., J.W., J.W.M., K.A.W.B., K.B.F., K.L.H.N., K.L.L., K.M.F., L.B.C., L.E.H., L.G.A., L.J.V.E., L.L.B., L.S.H., L.S.S., L.S.W., L.W.J., L.Y., M.A.A., M.D., M.E.M., M.Ganguli, M.Gearing, M.I.K., M.J., M.L., M.M., M.M.B., M.M.L., M.P.F., M.R., M.R.F., M.S., M.S.A., M.T., N.E.T., N.J.C., N.M.G., N.R.G.R., N.W.K., O.L.L., O.V., P.A.B., P.B.C., P.K., P.S., R.A.H., R.A.S., R.B., R.C.G., R.C.P., R.D., R.E.B., R.E.T., R.K., R.L.A., R.L.H., R.L.W., R.M.C., R.N.R., R.S., S.A., S.E.A., S.F., S.G., S.G.Y., S.L.C., S.S., S.Spina, S.T.A., S.Weintrub, T.B.G., T.D.B., T.G.B., T.J.M., T.M., T.M.F., V.M.V.D., W.A.K., W.G., W.J.M., W.W.P., W.W.S. Genotyping and expression studies: A.M.K., D.J.H., D.S., G.D.S., J.L., J.L.H., J.P.S., L.A.F., L.O., M.A.P.V., N.O.Z., R.M., V.K.S. Statistical analysis: B.D., D.Y., K.R., L.K., M.M.B., M.R.O

### COMPETING FINANCIAL INTEREST

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M. Hulette<sup>83</sup>, Bradley T. Hyman<sup>78</sup>, Gregory A. Jicha<sup>84</sup>, Lee-Way Jin<sup>85</sup>, Gyungah Jun<sup>38,86,87</sup>, M. Ilyas Kamboh<sup>3,88</sup>, Anna Karydas<sup>49</sup>, Jeffrey A. Kaye<sup>89,90</sup>, Ronald Kim<sup>91</sup>, Edward H. Koo<sup>71</sup>, Neil W. Kowall<sup>92,93</sup>, Joel H. Kramer<sup>94</sup>, Patricia Kramer<sup>89,95</sup>, Walter A. Kukull<sup>96</sup>, Frank M. LaFerla<sup>97</sup>, James J. Lah<sup>76</sup>, James B. Leverenz<sup>98</sup>, Allan I. Levey<sup>76</sup>, Ge Li<sup>99</sup>, Chiao-Feng Lin<sup>29</sup>, Andrew P. Lieberman<sup>100</sup>, Oscar L. Lopez<sup>89</sup>, Kathryn L. Lunetta<sup>87</sup>, Constantine G. Lyketsos<sup>101</sup>, Wendy J. Mack<sup>102</sup>, Daniel C. Marson<sup>58</sup>, Eden R. Martin<sup>28</sup>, Frank Martiniuk<sup>103</sup>, Deborah C. Mash<sup>104</sup>, Eliezer Masliah<sup>71,105</sup>, Ann C. McKee<sup>92,93</sup>, Marsel Mesulam<sup>106</sup>, Bruce L. Miller<sup>49</sup>, Carol A. Miller<sup>107</sup>, Joshua W. Miller<sup>86</sup>, Thomas J. Montine<sup>98</sup>, John C. Morris<sup>53,108</sup>, Jill R. Murrell<sup>67,75</sup>, Adam C. Naj<sup>28</sup>, John M. Olichney<sup>62</sup>, Joseph E. Parisi<sup>109,110</sup>, Elaine Peskind<sup>99</sup>, Ronald C. Petersen<sup>85</sup>, Aimee Pierce<sup>59</sup>, Wayne W. Poon<sup>63</sup>, Huntington Potter<sup>54</sup>, Joseph F. Quinn<sup>89</sup>, Ashok Raj<sup>54</sup>, Murray Raskind<sup>99</sup>, Eric M. Reiman<sup>112,113,114</sup>, Barry Reisberg<sup>69,115</sup>, Christiane Reitz<sup>25,82</sup>, John M. Ringman<sup>36</sup>, Erik D. Roberson<sup>58</sup>, Howard J. Rosen<sup>49</sup>, Roger N. Rosenberg<sup>116</sup>, Mary Sano<sup>51</sup>, Andrew J. Saykin<sup>67,117</sup>, Julie A. Schneider<sup>40,118</sup>, Lon S. Schneider<sup>57,119</sup>, William W. Seeley<sup>49</sup>, Amanda G. Smith<sup>54</sup>, Joshua A. Sonnen<sup>98</sup>, Salvatore Spina<sup>75</sup>, Robert A. Stern<sup>92</sup>, Rudolph E. Tanzi<sup>78</sup>, John Q. Trojanowski<sup>29</sup>, Juan C. Troncoso<sup>120</sup>, Debby W. Tsuang<sup>99</sup>, Otto Valladares<sup>29</sup>, Vivianna M. Van Deerlin<sup>29</sup>, Linda J. Van Eldik<sup>121</sup>, Badri N. Vardarajan<sup>38</sup>, Harry V. Vinters<sup>36,122</sup>, Jean Paul Vonsattel<sup>123</sup>, Li-San Wang<sup>29</sup>, Sandra Weintraub<sup>106</sup>, Kathleen A. Welsh-Bohmer<sup>15,124</sup>, Jennifer Williamson<sup>82</sup>, Randall L. Woltjer<sup>125</sup>, Clinton B. Wright<sup>126</sup>, Steven G. Younkin<sup>64</sup>, Chang-En Yu<sup>127</sup>, and Lei Yu<sup>40</sup>

<sup>1</sup>Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

<sup>2</sup>Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

<sup>3</sup>Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, USA.

<sup>4</sup>Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

<sup>5</sup>Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

<sup>6</sup>Gastroenterology and Liver Diseases, Pennsylvania State University, Hershey, Pennsylvania, USA.

<sup>7</sup>Department of Molecular and Clinical Cancer Medicine, National Institute for Health Research Liverpool Pancreas Biomedical Research Unit, Institute of Translational Medicine, University of Liverpool, Liverpool, UK.

<sup>8</sup>Department of Medicine A, University Medicine Greifswald, Greifswald, Germany.

<sup>9</sup>Aurora Health Care System, St. Luke's Medical Center, Milwaukee, Wisconsin, USA.

<sup>10</sup>Division of Gastroenterology, Hepatology and Nutrition, Virginia Commonwealth University Medical Center, Richmond, Virginia, USA.

<sup>11</sup>A full list of members and affiliations appears at the end of this paper, Alzheimer Disease Genetics Consortium.

<sup>12</sup>Department of Medicine, Saint Louis University, St. Louis, Missouri, USA.

<sup>13</sup>North Mississippi Medical Center, Tupelo, Mississippi, USA.

<sup>14</sup>Division of Gastroenterology, University of Michigan, Ann Arbor, Michigan, USA.

<sup>15</sup>Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA.

- <sup>16</sup>Division of Gastroenterology, Brigham and Women's Hospital, Boston, Massachusetts, USA.
- <sup>17</sup>Department of Medicine, Indiana University Medical Center, Indianapolis, Indiana, USA.
- <sup>18</sup>Digestive Disease Center, Medical University of South Carolina, Charleston, South Carolina, USA.
- <sup>19</sup>Department of Medicine, University of Utah Health Science Center, Salt Lake City, Utah, USA.
- <sup>20</sup>Department of Medicine/Biomedical Genetics Section, Boston University School of Medicine, Boston, Massachusetts, USA.
- <sup>21</sup>Department of Medicine, University of Florida, Gainesville, Florida, USA.
- <sup>22</sup>Dartmouth-Hitchcock Medical Center, Hanover, New Hampshire, USA.
- <sup>23</sup>Department of Molecular Physiology and Biophysics and Vanderbilt Center for Human Genetics Research, Vanderbilt University, Nashville, Tennessee, USA.
- <sup>24</sup>Division of Gastroenterology and Hepatology, Mayo Clinic, Jacksonville, Florida, USA.
- <sup>25</sup>Gertrude H. Sergievsky Center and Taub Institute on Alzheimer's Disease and the Aging Brain, Department of Neurology, Columbia University, New York, New York, USA.
- <sup>26</sup>Washington Hospital, Hagerstown, Maryland, USA.
- <sup>27</sup>Department of Internal Medicine, Yale University and Griffin Hospital, Derby, Connecticut, USA.
- <sup>28</sup>The John P. Hussman Institute for Human Genomics and Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami, Miami, Florida, USA.
- <sup>29</sup>Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.
- <sup>30</sup>University of Alabama at Birmingham, Birmingham, Alabama, USA.
- <sup>31</sup>Department of Epidemiology, Epidemiology Data Center, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA USA.
- <sup>32</sup>Department of Statistics, Carnegie Mellon University, Pittsburgh, Pennsylvania, USA.
- <sup>33</sup>Department of Neurology, Johns Hopkins University, Baltimore, Maryland, USA.
- <sup>34</sup>Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA.
- <sup>35</sup>Geriatric Research, Education and Clinical Center (GRECC), VA Ann Arbor Healthcare System (VAAHS), Ann Arbor, Michigan, USA.
- <sup>36</sup>Department of Neurology, University of California Los Angeles, Los Angeles, California, USA.
- <sup>37</sup>Department of Psychiatry, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA.
- <sup>38</sup>Department of Medicine (Genetics Program), Boston University, Boston, Massachusetts, USA.
- <sup>39</sup>Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, Fort Worth, Texas, USA.
- <sup>40</sup>Department of Neurological Sciences, Rush University Medical Center, Chicago, Illinois, USA.
- <sup>41</sup>Department of Behavioral Sciences, Rush University Medical Center, Chicago, Illinois, USA.
- <sup>42</sup>Civin Laboratory for Neuropathology, Banner Sun Health Research Institute, Phoenix, Arizona, USA.
- <sup>43</sup>National Alzheimer's Coordinating Center, University of Washington, Seattle, Washington, USA.

- <sup>44</sup>Rush Alzheimer's Disease Center, Rush University Medical Center, Chicago, Illinois, USA.
- <sup>45</sup>Department of Pathology, Northwestern University, Chicago, Illinois, USA.
- <sup>46</sup>Department of Neurology, University of Washington, Seattle, Washington, USA.
- <sup>47</sup>Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA.
- <sup>48</sup>Department of Psychiatry, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts, USA.
- <sup>49</sup>Department of Neurology, University of California San Francisco, San Francisco, California, USA.
- <sup>50</sup>Department of Neuroscience, Mount Sinai School of Medicine, New York, New York, USA.
- <sup>51</sup>Department of Psychiatry, Mount Sinai School of Medicine, New York, New York, USA.
- <sup>52</sup>Departments of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, New York, USA.
- <sup>53</sup>Department of Pathology and Immunology, Washington University, St. Louis, Missouri, USA.
- <sup>54</sup>USF Health Byrd Alzheimer's Institute, University of South Florida, Tampa, Florida, USA.
- <sup>55</sup>Department of Psychiatry, Vanderbilt University, Nashville, Tennessee, USA.
- <sup>56</sup>Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama, USA.
- <sup>57</sup>Department of Neurology, University of Southern California, Los Angeles, California, USA.
- <sup>58</sup>Department of Neurology, University of Alabama at Birmingham, Birmingham, Alabama, USA.
- <sup>59</sup>Department of Neurology, University of California Irvine, Irvine, California, USA.
- <sup>60</sup>Department of Psychiatry and Behavioral Sciences, Miller School of Medicine, University of Miami, Miami, Florida, USA.
- <sup>61</sup>Department of Psychiatry and Hope Center Program on Protein Aggregation and Neurodegeneration, Washington University School of Medicine, St. Louis, Missouri, USA.
- <sup>62</sup>Department of Neurology, University of California Davis, Sacramento, California, USA.
- <sup>63</sup>Institute for Memory Impairments and Neurological Disorders, University of California Irvine, Irvine, California, USA.
- <sup>64</sup>Department of Neuroscience, Mayo Clinic, Jacksonville, Florida, USA.
- <sup>65</sup>Wien Center for Alzheimer's Disease and Memory Disorders, Mount Sinai Medical Center, Miami Beach, Florida, USA.
- <sup>66</sup>Department of Neurology, Mayo Clinic, Jacksonville, Florida, USA.
- <sup>67</sup>Department of Medical and Molecular Genetics, Indiana University, Indianapolis, Indiana, USA.
- <sup>68</sup>Department of Neurology, Indiana University, Indianapolis, Indiana, USA.
- <sup>69</sup>Department of Psychiatry, New York University, New York, New York, USA.
- <sup>70</sup>C.S. Kubik Laboratory for Neuropathology, Massachusetts General Hospital, Charlestown, Massachusetts, USA.
- <sup>71</sup>Department of Neurosciences, University of California San Diego, La Jolla, California, USA.
- <sup>72</sup>Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia, USA.
- <sup>73</sup>Emory Alzheimer's Disease Center, Emory University, Atlanta, Georgia, USA.

<sup>74</sup>Neurogenetics Program, University of California Los Angeles, Los Angeles, California, USA.

<sup>75</sup>Department of Pathology and Laboratory Medicine, Indiana University, Indianapolis, Indiana, USA.

<sup>76</sup>Department of Neurology, Emory University, Atlanta, Georgia, USA.

<sup>77</sup>Division of Genetics, Department of Medicine and Partners Center for Personalized Genetic Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA.

<sup>78</sup>Department of Neurology, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts, USA.

<sup>79</sup>Center for Applied Genomics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA.

<sup>80</sup>Department of Pathology (Neuropathology), University of Pittsburgh, Pittsburgh, Pennsylvania, USA.

<sup>81</sup>Sanders-Brown Center on Aging, Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, Kentucky, USA.

<sup>82</sup>Taub Institute on Alzheimer's Disease and the Aging Brain, Department of Neurology, Columbia University, New York, New York, USA.

<sup>83</sup>Department of Pathology, Duke University, Durham, North Carolina, USA.

<sup>84</sup>Sanders-Brown Center on Aging, Department Neurology, University of Kentucky, Lexington, Kentucky, USA.

<sup>85</sup>Department of Pathology and Laboratory Medicine, University of California Davis, Sacramento, California, USA.

<sup>86</sup>Department of Biostatistics, Boston University, Boston, Massachusetts, USA.

<sup>87</sup>Department of Ophthalmology, Boston University, Boston, Massachusetts, USA.

<sup>88</sup>University of Pittsburgh Alzheimer's Disease Research Center, Pittsburgh, Pennsylvania, USA.

<sup>89</sup>Department of Neurology, Oregon Health & Science University, Portland, Oregon, USA.

<sup>90</sup>Department of Neurology, Portland Veterans Affairs Medical Center, Portland, Oregon, USA.

<sup>91</sup>Department of Pathology and Laboratory Medicine, University of California Irvine, Irvine, California, USA.

<sup>92</sup>Department of Neurology, Boston University, Boston, Massachusetts, USA.

<sup>93</sup>Department of Pathology, Boston University, Boston, Massachusetts, USA.

<sup>94</sup>Department of Neuropsychology, University of California San Francisco, San Francisco, California, USA.

<sup>95</sup>Department of Molecular & Medical Genetics, Oregon Health & Science University, Portland, Oregon, USA.

<sup>96</sup>Department of Epidemiology, University of Washington, Seattle, Washington, USA.

<sup>97</sup>Department of Neurobiology and Behavior, University of California Irvine, Irvine, California, USA.

<sup>98</sup>Department of Pathology, University of Washington, Seattle, Washington, USA.

- <sup>99</sup>Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA.
- <sup>100</sup>Department of Pathology, University of Michigan, Ann Arbor, Michigan, USA.
- <sup>101</sup>Department of Psychiatry, Johns Hopkins University, Baltimore, Maryland, USA.
- <sup>102</sup>Department of Preventive Medicine, University of Southern California, Los Angeles, California, USA.
- <sup>103</sup>Department of Medicine - Pulmonary, New York University, New York, New York, USA.
- <sup>104</sup>Department of Neurology, University of Miami, Miami, Florida, USA.
- <sup>105</sup>Department of Pathology, University of California San Diego, La Jolla, California, USA.
- <sup>106</sup>Cognitive Neurology and Alzheimer's Disease Center, Northwestern University, Chicago, Illinois, USA.
- <sup>107</sup>Department of Pathology, University of Southern California, Los Angeles, California, USA.
- <sup>108</sup>Department of Neurology, Washington University, St. Louis, Missouri, USA.
- <sup>109</sup>Department of Anatomic Pathology, Mayo Clinic, Rochester, Minnesota, USA.
- <sup>110</sup>Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA.
- <sup>111</sup>Department of Neurology, Mayo Clinic, Rochester, Minnesota, USA.
- <sup>112</sup>Arizona Alzheimer's Consortium, Phoenix, Arizona, USA.
- <sup>113</sup>Banner Alzheimer's Institute, Phoenix, Arizona, USA.
- <sup>114</sup>Neurogenomics Division, Translational Genomics Research Institute, Phoenix, Arizona, USA.
- <sup>115</sup>Alzheimer's Disease Center, New York University, New York, New York, USA.
- <sup>116</sup>Department of Neurology, University of Texas Southwestern, Dallas, Texas, USA.
- <sup>117</sup>Department of Radiology and Imaging Sciences, Indiana University, Indianapolis, Indiana, USA.
- <sup>118</sup>Department of Pathology (Neuropathology), Rush University Medical Center, Chicago, Illinois, USA.
- <sup>119</sup>Department of Psychiatry, University of Southern California, Los Angeles, California, USA.
- <sup>120</sup>Department of Pathology, Johns Hopkins University, Baltimore, Maryland, USA.
- <sup>121</sup>Sanders-Brown Center on Aging, Department of Anatomy and Neurobiology, University of Kentucky, Lexington, Kentucky
- <sup>122</sup>Department of Pathology & Laboratory Medicine, University of California Los Angeles, Los Angeles, California, USA.
- <sup>123</sup>Taub Institute on Alzheimer's Disease and the Aging Brain, Department of Pathology, Columbia University, New York, New York, USA.
- <sup>124</sup>Department of Psychiatry & Behavioral Sciences, Duke University, Durham, North Carolina, USA.
- <sup>125</sup>Department of Pathology, Oregon Health & Science University, Portland, Oregon, USA.
- <sup>126</sup>Evelyn F. McKnight Brain Institute, Department of Neurology, Miller School of Medicine, University of Miami, Miami, Florida, USA.
- <sup>127</sup>Department of Medicine, University of Washington, Seattle, Washington, USA.

## Abstract

Pancreatitis is a complex, progressively destructive inflammatory disorder. Alcohol was long thought to be the primary causative agent, but genetic contributions have been of interest since the discovery that rare *PRSS1*, *CFTR*, and *SPINK1* variants were associated with pancreatitis risk. We now report two significant genome-wide associations identified and replicated at *PRSS1-PRSS2* ( $1 \times 10^{-12}$ ) and x-linked *CLDN2* ( $p < 1 \times 10^{-21}$ ) through a two-stage genome-wide study (Stage 1, 676 cases and 4507 controls; Stage 2, 910 cases and 4170 controls). The *PRSS1* variant affects susceptibility by altering expression of the primary trypsinogen gene. The *CLDN2* risk allele is associated with atypical localization of claudin-2 in pancreatic acinar cells. The homozygous (or hemizygous male) *CLDN2* genotype confers the greatest risk, and its alleles interact with alcohol consumption to amplify risk. These results could partially explain the high frequency of alcohol-related pancreatitis in men – male hemizygous frequency is 0.26, female homozygote is 0.07.

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The exocrine pancreas is a simple digestive gland of only two primary cell types, each with a single function (Supplementary Figure 1). Recurrent acute pancreatic inflammation can, but does not always, progress to irreversible damage of the gland, including fibrosis, atrophy, pain, and exocrine and endocrine insufficiency,<sup>1-3</sup> known as chronic pancreatitis. Different genetic and environmental factors produce the same clinical phenotype<sup>4</sup>.

We collected biological samples and phenotypic data from 1000 patients with recurrent acute pancreatitis and chronic pancreatitis plus controls in the North American Pancreatitis Study 2 (NAPS2)<sup>5</sup>. The primary environmental risk factor identified was heavy alcohol drinking when symptoms of pancreatitis began, based on the assessment of the study physician, called here alcohol-related pancreatitis.

To further define genetic risk, we conducted a two-stage (discovery/replication) genomewide association study (GWAS). The final data set for the Stage 1 cohort included 676 chronic pancreatitis cases and 4507 controls of European ancestry (Supplementary Figs. 2-3) genotyped at 625,739 SNPs (Table 1; Supplementary Table 1). Genomewide significant associations ( $p\text{-value} < 5 \times 10^{-8}$ ) were identified at two loci. The most highly associated SNP fell in Xq23.3, dubbed the *CLDN2* locus, the other in 7q34, the *PRSS1-PRSS2* locus (Fig. 1; Table 2; Supplementary Figs. 4-5, Supplementary Table 2). *CLDN2* encodes the protein claudin-2, while *PRSS1* encodes cationic trypsinogen, and *PRSS2* encodes anionic trypsinogen.

The Stage 2 cohort included 910 cases (331 chronic pancreatitis, 579 recurrent acute pancreatitis; Table 1, Supplementary Table 1), again genotyped at 625,739 SNPs, and 4170 controls, most genotyped previously on the Illumina 1M. All subjects were of European ancestry as determined by genetic analyses. Recurrent acute pancreatitis and chronic pancreatitis were modeled as having common susceptibilities, with chronic pancreatitis occurring over time in the presence of additional disease-modifying factors.<sup>6</sup> It is possible that this assumption reduces power relative to a study comprising solely chronic pancreatitis or recurrent acute pancreatitis cases. Our primary targets in Stage 2 were the *PRSS1-PRSS2* and *CLDN2* loci, although we also conducted a joint analysis<sup>7</sup> of Stage 1 and Stage 2 data to uncover any new risk loci. After controlling for ancestry, these data demonstrated significant effects for the *CLDN2* and *PRSS1-PRSS2* loci (Figure 1; Supplementary Table 2-3; Supplementary Figs. 6-7). Quality of SNP genotypes supported the association (Supplementary Fig. 8). The frequencies of the putative risk alleles at these 2 loci were 0.57 for the C allele at rs10273639 (*PRSS1-PRSS2* locus), with the minor T allele reducing risk, and 0.26 for the T allele at rs12688220 (*CLDN2* locus). No other locus shows association after accounting for SNP genotype quality (Supplementary Figs. 6-8).



*PRSS1* gain-of-function mutations, such as p.R122H, increase risk for recurrent acute pancreatitis and chronic pancreatitis<sup>8</sup>, as do increased copy number<sup>9,10</sup>. Rare loss-of-function mutations in *PRSS2* are protective<sup>11</sup>. However, rs10273639 is in the 5' promoter region of *PRSS1*. Because it is the only highly associated SNP in the locus, we validated its genotypes by independent TaqMan genotyping and also genotyped two SNPs in linkage disequilibrium with it (footnote, Supplementary Table 4)<sup>12,13</sup>. We screened *PRSS1* for rare variants in 1138 subjects: 418 chronic pancreatitis, 350 recurrent acute pancreatitis, and 379 controls. Three known disease-associated variants (A16V, N29I, R122H) were identified in 23 subjects (Supplementary Table 4). These gain-of-function variants occur almost solely in cases (22 out of 23), and two of them, A16V and R122H, likely fall on the C or risk haplotype of this locus (Supplementary Table 4). Nonetheless, with only 19 A16V and R122H events in cases, these rare alleles cannot account for the association observed at this locus.

Sixty-nine control pancreas tissue samples from three sources were genotyped at rs10273639, and cDNA was used to quantify *PRSS1* and control gene expression (Supplementary Table 5). For all three sets of quantitative PCR data, the slope relating count of genotype C allele to *PRSS1* expression level was positive; together, the samples provide evidence ( $p = 0.01$ ) that alleles at rs10273639 affect expression of *PRSS1*: expression levels were highest in patients with two C alleles at rs10273639, intermediate in heterozygotes, and lowest in subjects with two T alleles. Based on this evidence, we posit that reduced trypsinogen production protects the pancreas from injury, as has been observed in genetic mouse models<sup>14</sup>.

*CLDN2* is considered the primary candidate gene within our *CLDN2* locus. Claudin-2 is attractive because it serves as a highly regulated tight junction protein forming low-resistance, cation-selective ion and water channels between endothelial cells<sup>15,16</sup> and is normally expressed at low levels between cells of the pancreatic ducts and in pancreatic islets<sup>17,18</sup>. The *CLDN2* promoter includes an NF $\kappa$ B binding site<sup>19</sup>, and gene expression is enhanced in other cells under conditions associated with injury or stress<sup>20-22</sup>. Claudin-2 can also be expressed by acinar cells when stressed, as reported in porcine models of acute pancreatitis<sup>23</sup>. Other genes within the *CLDN2* locus include *MORC4*, *RIPPLY1*, and *TBC1D8B*. *MORC4* is expressed at low levels in most tissues, including the pancreas, with higher levels in the placenta and testis<sup>24</sup>. The *MORC4* protein contains a CW four-cysteine zinc-finger motif, nuclear localization signal, and nuclear matrix-binding domain, suggesting that it may be a transcription factor<sup>24</sup>, but its expression does not appear to correlate with pancreatitis (Supplementary Fig. 9). *RIPPLY1* and *TBC1D8B* are not known to be expressed in the pancreas.

To our knowledge, genetic variations in *CLDN2* have never been associated with disease in humans. We assessed DNA sequence variants around *CLDN2*, RNA, and protein expression for claudin-2 in control tissue classified by histology and genotype (Supplementary Table 6, Supplementary Fig. 10). Evaluating 1000 Genomes data, no exonic variation was identified that could explain the association signal. Using materials and methods described previously for *PRSS1* expression, *CLDN2* expression levels in control tissues did not correlate with the *CLDN2* locus risk genotype ( $p$ -value = 0.32). Protein was extracted from the tissue, and only one protein band of the appropriate size was observed with anti-claudin-2 antibodies on Western blot, which correlated with tissue inflammation as determined by systematic grading of histology in adjacent tissue (Fig. 2A, Supplementary Fig. 10). Immunohistochemical staining with anti-claudin-2 antibodies was verified in normal tissue (Fig. 2B), with kidney, duodenum, and bile ducts serving as additional positive controls (not shown). Protein localization was assessed in 12 GWAS cases who underwent pancreatic surgery: 6 with the *CLDN2*-containing high-risk genotype and 6 without. Claudin-2

cytoplasmic granular staining was markedly increased in both duct and acinar cells in chronic pancreatitis cases (Fig. 2C-E). Only chronic pancreatitis cases with the high-risk *CLDN2* genotype demonstrated moderate-to-strong claudin-2 staining along the basolateral membrane of acinar cells (Fig. 2D, 2E, Supplementary Table 6). Claudin-2 was also expressed in macrophages, which could contribute to the pathologic inflammatory process<sup>25</sup> (Fig. 2C, F).

Most studies report excessive alcohol consumption as the major risk factor for adult-onset chronic pancreatitis<sup>26-29</sup>. However, only 3% of patients who are alcoholics develop chronic pancreatitis<sup>30</sup>, suggesting a pancreas-targeting risk factor. We compared genotypes based on whether pancreatitis was alcohol-related (yes/no)<sup>5,31</sup>. Setting control genotypes counts as the baseline category to be compared with case genotypes, the jointly estimated odds ratios for cases with a positive alcohol-related pancreatitis was greater for both rs10273639 (*PRSSI-PRSS2* locus) and rs12688220 (*CLDN2* locus) than those estimated for cases with a negative alcohol-related pancreatitis (Table 3). Thus, the effects of both loci appeared to be amplified by alcohol consumption. In a *case-only analysis*, both loci appear to interact with alcohol-related pancreatitis (Table 3), the *CLDN2* locus most prominently (p-value =  $4 \times 10^{-7}$ ).

We conclude that a common allele in the *PRSSI-PRSS2* locus is associated with lower *PRSSI* gene expression and that this effect is independent of the previously reported rare gain-of-function *PRSSI* variants that increase susceptibility to both recurrent acute pancreatitis and chronic pancreatitis<sup>8</sup>. For this reason, and because risk variants at the *PRSSI-PRSS2* locus exert a similar effect in patients with recurrent acute pancreatitis or chronic pancreatitis, it is reasonable to conjecture that variation at rs10273639 or variation in linkage disequilibrium with it directly affects risk for chronic pancreatitis and recurrent acute pancreatitis through its impact on trypsinogen expression. Variation at the *CLDN2* locus, however, is much more strongly associated with chronic pancreatitis than recurrent acute pancreatitis, suggesting that it likely acts as a disease modifier to accelerate transition from recurrent acute pancreatitis to chronic pancreatitis. The significant association of the *CLDN2* locus with alcohol suggests that the high-risk allele in the *CLDN2* locus may modify risk through a non-trypsin-dependent process. Thus, we have characterized two common genetic risk modifiers for sporadic and alcohol-related chronic pancreatitis.

## Online Methods

### Subject recruitment

Details of recruitment of cases and controls are reported in Supplementary Table 1. All studies were conducted under institutional review board-approved protocols.

### Stage 1 samples

All N = 758 Stage 1 case samples were from the North American Acute Pancreatitis Study (NAPS2<sup>5</sup>) were diagnosed with chronic pancreatitis, and were characterized for alcohol-related pancreatitis (Table 1). chronic pancreatitis occurs in less than 0.05% of the population, so a convenience sample provides essentially identical power as a same-sized sample of controls selected for the absence of chronic pancreatitis<sup>32</sup>. For controls, we used genotypes from 4076 cases and controls from the Alzheimer Disease Genetics Consortium (ADGC) and 493 NAPS2 subjects, all genotyped on the same platform as the chronic pancreatitis samples.

## Stage 2 samples

The Stage 2 samples consisted of N=343 chronic pancreatitis and N=627 recurrent acute pancreatitis cases (Table 1, Supplementary Table 1) as well as 4191 control subjects (3986 from the NeuroGenetics Research Consortium, NGRC, and 205 NAPS2 controls).

## Genotypes

All cases and NAPS2 controls were genotyped by the University of Pittsburgh Genomics and Proteomics Core Laboratories using the Illumina HumanOmniExpress Beadchip. Samples were processed and scanned using the manufacturer's recommended protocols with no modifications. ADGC samples<sup>33</sup> were also genotyped using Illumina HumanOmniExpress Beadchips, whereas NGRC samples<sup>34</sup> were genotyped on the Illumina Human1M-Duo DNA Analysis BeadChip.

## Quality Control (QC) for Stage 1

QC was performed for individuals and then SNPs to determine which samples and SNPs should not be included in the analysis ("dropped"). Assessing sex miscalls based on  $\times$  chromosome genotypes using Plink<sup>35</sup>, 7 chronic pancreatitis cases and 20 controls (10 NAPS2; 10 ADGC) were dropped. Based on the requirement for 95% complete genotypes per individual, 40 cases and 27 controls (20 NAPS2 controls and 7 ADGC controls) were dropped. Searching duplicate or highly related samples based on genotype and using GCTA software<sup>36</sup> (Genetic Relationship Matrix score GRM > 0.4), 35 cases and 78 controls (2 NAPS2, 76 ADGC) were dropped. After these QC filters, 676 cases and 4507 controls remained for association analysis.

SNP QC was first performed using NAPS2 and ADGC samples separately. Ancestry was estimated using dacGem<sup>37</sup> based on 9700 SNPs that had a genotype completion rate of 99.9%, a minor allele frequency MAF  $\geq$  0.05, and were separated by at least 500Kb. Analysis of genotypes from NAPS2 subjects identified 1 significant dimensions of ancestry and clustered subjects into 3 groups (Supplementary Fig. 1). Groups A and B, illustrated in Supplementary Figure 1, delineate 764 and 282 subjects, respectively, of European ancestry (self-identified); SNP QC for MAF and Hardy Weinberg Equilibrium (HWE) were performed on data from these subjects. Of 731,442 SNPs received, 633,790 passed QC filters. SNPs were dropped for the following reasons: 3165 for map location; 11,977 for call rate; 77,300 for MAF < 0.01; and 5219 failed HWE (p-value < 0.005).

ADGC data were received in three waves of 1763, 1110, and 1266 subjects. In the first wave, 659,224 SNPs were received, while in waves two and three, 730,525 SNPs were received. After QC as described for the chronic pancreatitis cohort, including harmonization with SNPs passing QC in the chronic pancreatitis cohort, 604,059, 632,761, and 633,023 SNPs remained, respectively. After merging cohorts, 30 related subjects were dropped, leaving 4046 ADGC subjects. Of the 633,615 unique SNPs in this ADGC, QC filters dropped 5 for low MAF and 5316 for HWE, leaving 628,294 SNPs. Combining ADGC and chronic pancreatitis cohorts and performing another round of QC yielded 625,739 SNPs for analysis.

## QC for Stage 2

QC for individuals was performed as described for Stage 1. These individual-specific QC filters removed 60 cases, leaving 331 chronic pancreatitis and 579 recurrent acute pancreatitis cases for analysis; 14 controls were also removed, leaving 4177 controls for analysis. We analyzed all SNPs passing QC at Stage 1.

## Association analysis

To control confounding due to ancestry, the first 10 major eigenvectors from the spectral decomposition were used as covariates in Stage 1 and Stage 2 analyses<sup>38</sup>, although only one was significant. We contrasted the genotypes of case subjects and controls via logistic regression and a log-additive (logit) model using Plink<sup>35</sup>. Genotypes for any SNPs showing association p-value  $< 5 \times 10^{-7}$  were manually inspected for valid genotype clustering. SNPs showing poor-quality clustering were excluded. Following Skol *et al.*<sup>7</sup> and others, we take an overall significance level of  $5 \times 10^{-8}$  and  $5 \times 10^{-7}$  for strongly suggestive association.

To determine whether alcohol interacts with genetic variation to alter risk of pancreatitis, data from cases were fit to a general linear model in which count of alleles or genotypes predicted alcohol etiology (yes/no). The test statistic was obtained as a likelihood ratio chi-square. Note that in these analyses and any analyses other than genomewide association, we model the male genotypes as 0 and 2<sup>39,40</sup>. For the genomewide association, Plink encodes the count of minor alleles in males as 0 and 1 and includes a sex effect, but the 0/2 encoding for males is a more powerful approach<sup>39,40</sup>.

## DNA extraction

DNA was obtained using standard methods<sup>41</sup>.

## Pancreatic tissue processing

Tissue was obtained from two sources [Pitt and Pancreatic Adenocarcinoma Gene-Environment Risk (PAGER) from the University of Pittsburgh and PSU from Pennsylvania State University] and processed in three batches: banked (Pitt) and prospectively collected (PAGER) surgical waste from uninvolved pancreas and normal pancreas specimens from the Gift of Life Program that were not used for transplantation (PSU). PAGER samples were snap-frozen, placed in RNAlater solution (Ambion), and stored at  $-80^{\circ}\text{C}$ . PSU pancreas samples were also snap frozen and preserved in formalin or placed in RNAlater solution. RNA was isolated using Trizol reagent (Invitrogen), and its quality examined in 1% agarose gel stained with ethidium bromide. cDNA was transcribed using oligo dt primers and the Superscript II reverse transcriptase kit (Invitrogen).

## Gene Expression

Relative expression of *PRSS1*, *PRSS2*, *CTRC*, and *18S* was determined by analyzing cDNA using Taqman®-based rtPCR assays (Applied Biosystems). Raw absolute quantitation results were analyzed and converted to relative expression results by software packages SDS V2.3 and DataAssist V1.0 (Applied Biosystems). Assays were repeated in triplicate or quadruplicate. Three sets of samples were assessed, two from Pitt (N=10 and 22) and one from PSU (N=37). PSU results were normalized against 18S, Pitt against CTRC. From each of these three data sets, mean gene expression per sample was regressed against allele count to obtain an estimated slope, standard error, and z-score. We then calculated an overall z-score as a weighted average of the individual z-scores, with weights determined by sample size.

## Antibodies

Antibodies against claudin proteins (Invitrogen) were assessed using Western blot for mouse anti-claudin-2 (Catalog No. 32-5600), mouse anti-claudin-4 (Catalog No. 32-9400), and mouse alpha-tubulin antibody (Catalog no. AA12.1 The Developmental Studies Hybridoma Bank at the University of Iowa, <http://dshb.biology.uiowa.edu/Antibody-list>). Immunohistochemistry was performed using monoclonal antibodies for claudin-2 (Catalog #32-5600, 1:1,000 dilution). Immunofluorescence was performed using mouse anti-claudin-2

(Catalog No. 32-5600) and goat anti-human CD68 (Catalog #sc-7082, Santa Cruz Biotechnology Inc.). The secondary antibodies for Immunofluorescence were goat anti-mouse CY3 and anti goat Cy5 from Jackson ImmunoResearch.

### SDS-PAGE and WESTERN Blotting

Protein homogenates for Western blotting were obtained from snap-frozen tissue that was homogenized and sonicated in lysis buffer supplemented with protease inhibitors. Protein concentration was determined by the Bradford method using a kit from Bio-Rad. Proteins were separated on 12% SDS-PAGE<sup>42</sup> followed by transfer to polyvinylidene difluoride (PVDF) membranes<sup>43</sup>, for Western blotting<sup>44</sup>. Immunodetection of bound antibodies on PVDF membrane was performed using ECL reagents (Amersham Biosciences). All procedures were carried out according to manufacturer instructions.

### Immunohistochemistry

Standard automated immunohistochemistry was performed for claudin 2 (antibodies listed above) on formalin-fixed, paraffin-embedded, 5 micron-thick tissue sections. Following deparaffinization in xylene and rehydration in ethanol, antigen retrieval was performed using EDTA pH8 buffer. The Dako Autostainer Plus was used; the slides were incubated for 30 minutes with the primary antibodies, followed by incubation with the secondary reagent (Mach 2 Mouse HRP Polymer from Biocare Medical) for 30 minutes. The chromogen was developed (Dako DAB+) for 10 minutes. The immunohistochemical stains were reviewed by one of the authors (A.M.K.). Cytoplasmic, granular, and membranous staining, predominantly in the lateral cell membranes, were graded on an intensity scale of 0-4 (0, negative; 1, weak; 2, moderate; 3, strong). The staining intensity was very patchy from lobule to lobule in most cases.

### Immunofluorescence

Cryostat sections (5 micron) of pancreas were washed 3 times with phosphate-buffered saline (PBS), followed by 3 washes with solution of .5% BSA in PBS. Sections were blocked with 2% BSA solution for 30 minutes. The slides were incubated for 1 hour at room temperature with primary antibody for claudin-2 1:100 and goat anti-human CD68 in 0.5% BSA solution. Slides were washed 3 times with BSA solution and incubated for 1 hour at 20°C with 1:500 dilution anti-goat CY5 and 1:1000 dilution goat anti-mouse CY3 secondary antibodies in BSA solution. Nuclei were stained with Hoeschts dye (bisbenzamide 1mg/100ml water) for 30 seconds. After 3 rinses with PBS, sections were cover slipped with Gelvatol mounting media. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (software version 1.7a). The Cy5 signal (CD68) was pseudocolored as green to show colocalization with the red Claudin signal as yellow.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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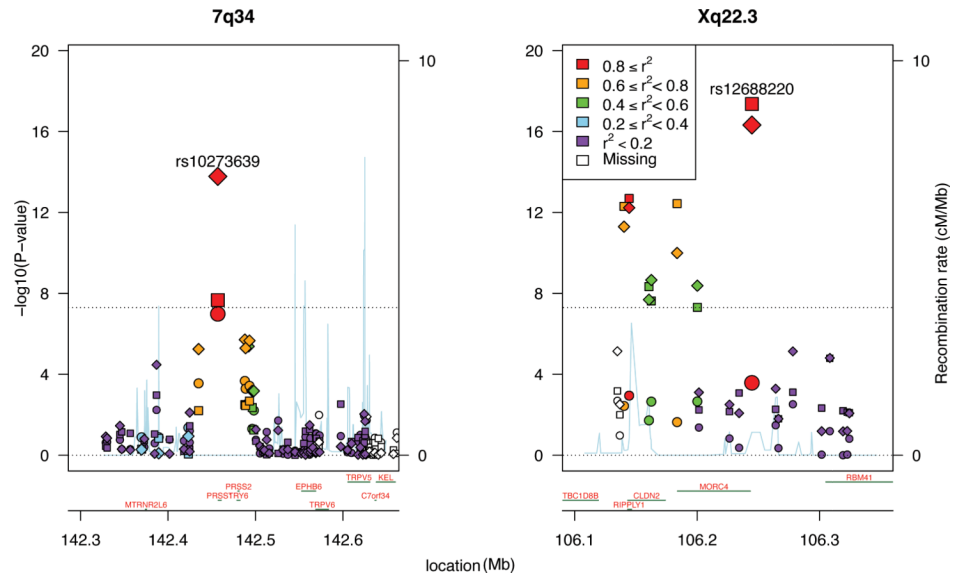
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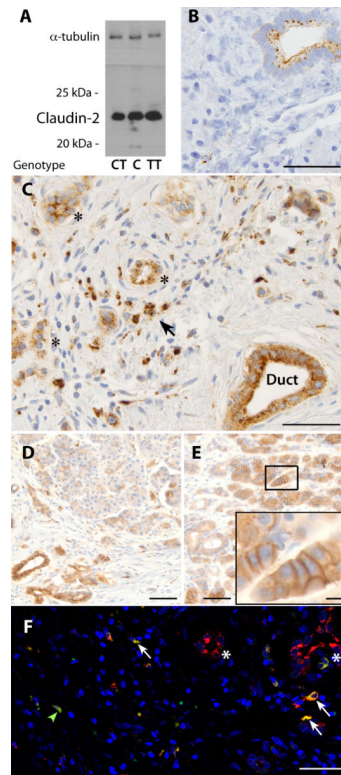
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**Figure 1.** Manhattan plot showing the negative log (base 10) of the p-value for the association of SNP genotype with affection status for all SNPs passing quality control filters and falling within a selected region of the *PRSS1-PRSS2* and *CLDN2* loci. Regions selected to highlight the most associated SNPs. Squares indicate Stage 1 results, circles for Stage 2, diamonds for combined Stage 1 and 2 data. After accounting for the most highly associated SNP at each locus, no other SNP approached genomewide-significant association.



**Figure 2.**

Expression and localization of claudin-2 in the human pancreas using mouse anti-claudin-2 antibodies based on rs12688220 genotype. **A.** Western blot of anti-claudin-2 antibody from 3 control samples genotyped at rs12688220 (TT is high risk). The antibody reacts with a protein at ~22-23 kDa, consistent with claudin-2. Samples had inflammation and/or fibrosis on histology of adjacent tissue.  $\alpha$ -tubulin, loading control. Blots from all controls are presented in Supplementary Figure 8. **B.** Anti-claudin-2 staining (brown color) of normal-appearing control tissue localizing to ducts but not to acinar cells (scale bar=50 $\mu$ m). **C.** Severe chronic pancreatitis from a case with the high-risk (T male or TT female) genotype. Claudin-2 staining localizes to the intralobular duct (Duct), atrophic acini (\*), and cells with morphologic appearance of macrophages (arrow)(scale bar=50 $\mu$ m). **D.** Chronic pancreatitis tissue from a patient with the low-risk genotype (CC or CT) with staining localizing to the duct and granular staining in acinar cells (scale bar = 100  $\mu$ m). **E.** Chronic pancreatitis, high-risk genotype with intense staining of acinar cell basolateral membrane (scale bar=100  $\mu$ m, enlarged in inset, scale bar=10 $\mu$ m). **F.** Immunofluorescence staining of control human pancreatic tissue claudin-2 staining (red) localizing to the ducts (\*) and co-localizing with the macrophage marker CD68 (green, colocalized with red is yellow, arrows). Nuclei stained with Hoechst's dye, blue, scale bar = 100 $\mu$ m).

Table 1

Characterization of case subjects used for GWAS.\*

	chronic pancreatitis	recurrent acute pancreatitis	chronic pancreatitis + recurrent acute pancreatitis
Stage 1	676	--	676
Alcohol-related pancreatitis †	264	--	264
Yes			
No	411	--	411
Unknown	1	--	1
Stage 2	331	579	910
Alcohol-related pancreatitis †	70	113	183
Yes			
No	256	462	718
Unknown	5	4	9
Combined	930	579	1506
Alcohol-related pancreatitis †	334	113	447
Yes			
No	667	462	1129
Unknown	6	4	10

\* Does not include information from controls in Stage 1 (n=4514) or from Stage 2 (n=4053). For more complete characterization of samples, please see Supplementary Table 1 in the Appendix.

† Alcohol-related pancreatitis was assigned by the study physician at enrollment.

**Table 2**

Results for leading SNPs at the *PRSS1-PRSS2* and *CLDN2* loci from Stage 1, Stage 2, and joint analysis.

CHR	SNP	BP	A1*	A2	Allele Frequency (A1)		Stage 1			Stage 2			Combined		
					cases	controls	OR	se(OR)	P	OR	se(OR)	P	OR	se(OR)	P
7	rs10273639	142456928	T	C	0.350	0.424	0.712	0.044	$3.0 \times 10^{-8}$	0.748	0.039	$7.5 \times 10^{-8}$	0.734	0.029	$2.0 \times 10^{-14}$
X	rs7057398	106144529	C	T	0.374	0.281	1.493	0.075	$1.4 \times 10^{-15}$	1.210	0.066	$1.8 \times 10^{-5}$	1.321	0.049	$4.6 \times 10^{-17}$
X	rs12688220	106244767	T	C	0.367	0.261	1.612	0.081	$2.4 \times 10^{-21}$	1.238	0.073	$2.3 \times 10^{-6}$	1.385	0.054	$2.3 \times 10^{-22}$

\* A1 is the allele counted for purposes of computing odds ratio and associated statistics. The model used here includes covariates to control for the two leading eigenvectors for ancestry, as was done in the Pink analyses, but differs in its treatment of the minor allele count for the *CLDN2* locus, which resides on the X chromosome (as described in Online Methods). Alleles given are refSNP alleles according to dbSNP. See Supplementary Table 2 for all SNPs passing quality control and showing p-value  $< 5 \times 10^{-7}$  for Stage 1 or Stage 2 or the joint analysis.

**Table 3**

Allele frequencies for rs10273639 (risk allele C) and rs12688220 (risk allele T) when data are stratified by controls or pancreatitis ± alcohol-related diagnosis.

Status	Alcohol-related	Number of individuals	rs10273639 <sup>I</sup> (C) frequency	rs12688220 <sup>I</sup> (T) frequency
Control	--	8029	0.576	0.261
Pancreatitis	No	1129	0.634	0.322
	Yes	447	0.696	0.427

<sup>I</sup>Using data from *cases only* and in a joint analysis of both SNPs, rs12688220 predicts alcohol-related pancreatitis as genotypes ( $\chi^2=29.57$ ; DF=2; p-value =  $4 \times 10^{-7}$ ) or count of risk alleles ( $\chi^2=13.17$ ; DF=1; p-value =  $3 \times 10^{-4}$ ). rs10273639 (*PRSS1-PRSS2* locus) is a significant predictor (count of risk alleles:  $\chi^2=5.68$ ; DF=1; p-value = 0.017; genotypes:  $\chi^2=6.05$ ; DF=2; p-value = 0.049), even after accounting for the effects of rs12688220.